IMPLICATIONS OF EMBRYO DESICCATION TOLERANCE, SEED DORMANCY, AND SEED DAMAGE FOR CONSERVATION OF *PRITCHARDIA* PALMS ENDEMIC TO HAWAI'I

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DEDICATION

This dissertation is dedicated to the memory of Amparo Claramunt. Her blessings allowed me to pursue my ambitions.

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ABSTRACT

Pritchardia remota (Kuntze) Becc. is an endemic Hawaiian palm. This species is listed as endangered due to threats such as invasive plants and animals, stochastic events, and/or reduced reproductive vigor. However, *P. remota* seeds are not presently stored in genebanks because it is presumed that they do not remain viable in storage. Yet, there are no studies that report the tolerance of *Pritchardia* species to storage conditions. Assessing the desiccation tolerance of *Pritchardia* is hampered by their difficult physiology. Germination is delayed in seeds of *Pritchardia* species and the mechanisms that control these delays are not understood. Clarifying these mechanisms can assist in forming propagation protocols for the creation of genetically diverse populations useful for recovery actions. Moreover, introduced vertebrate predators are known to damage seeds of *Pritchardia*. It is not known if *Pritchardia* seeds can tolerate damage and remain viable.

The studies reported here show that developmental patterns in *P. remota* are intermediate to those observed for species that can and can not tolerate storage in genebanks. Embryos tolerate relatively high levels of drying and remain viable. However, they do not tolerate the extreme drying (< 0.10 g H_20/ g dry weight) necessary to maintain viability in conventional genebanks conditions. Therefore, it may be necessary to employ other techniques, such as cryo-preservation, to extend the longevity of this species in storage.

Pritchardia remota seed are dormant at shedding. Embryo growth is restricted by the operculum and endocarp. Embryos are also underdeveloped and must reach a critical length, moisture content, and water potential for operculum displacement and subsequent

radicle protrusion. Germination is hastened at warm constant temperatures (*i.e.*, $\geq$ 25°C) and if seeds are removed from the fruit coats. The combination of mechanisms suggests that *P. remota* seeds are morpho-physiologically dormant.

Finally, moderate levels of seed damage may not be limiting in terms of germination for *Pritchardia* species. Damage to the endosperm caused seeds to germinate more rapidly compared to non-damaged seeds. Nevertheless, damaged seeds may succumb to fungal attack. Therefore, it is critical therefore to prevent predators from damaging *Pritchardia* seeds.

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CHAPTER 1

INTRODUCTION

Arecaceae, or Palmae, is a diverse family of over 2500 species, occurring predominantly in sub-tropical and tropical zones. Many species, such as the oil palm (*Elaeis guineensis* Jacq.) or coconut (*Cocos nucifera L.*), are highly valued commercially because of the resources that can be extracted from them and used for various industrial purposes (Uhl and Dransfield 1987; Hong *et al.* 1998). Additionally, many indigenous peoples rely on palms to provide food, shelter, and fiber (Uhl and Dransfield 1987). Moreover, palms are important in the horticultural industry. For example, numerous palm species are used to grace our interior and exterior landscapes. For these reasons, Arecaceae ranks with Fabaceae (*i.e.*, legumes) and Poaceae (*i.e.*, grains and cereals) as one of the most economically important plant families on a global scale.

Aside from their economic and ethno-botanical significance, palms also provide vital ecological functions (Uhl and Dransfield 1987). For example, palms often house vast arthropod communities and vertebrates use their fruits as sustenance. Riparian palms, like *Nypa* spp., function to stabilize stream banks. Finally, *Pritchardia remota* (Kuntze) Becc. affords nesting and loafing sites for several types of ocean going birds (U.S.F.W.S. 1998).

Yet, as with many wild plants, numerous palm species are threatened with extinction due to habitat loss, habitat fragmentation, and invasive alien species. This is most evident for palms in tropical areas. Given the significance of palms one could conjecture that methods to conserve the genetic diversity of palms *ex situ* are well known. However, seed storage characteristics and germination behavior have been studied in

detail for *c.* 5% of palm species (Davies and Pritchard 1998; Wood and Pritchard 2003). This lack of knowledge applies to Hawaii's only native genus of palms, *Pritchardia*, as well.

Pritchardia remota is one of 19 endemic Hawaiian *Pritchardia* species (Wagner *et al.* 1999). It is a solitary palm; *c.* 4-5 m tall, with unarmed petioles, and costa-palmate leaves. *P. remota* only occurs naturally in scattered groves on Nihoa, a small, uninhabited island approximately 250 km northwest of Honolulu, Hawaii. A total of 680 individuals of different age classes remain. Early Polynesian contact on Nihoa may have reduced the total number of individuals. Currently, threats to these populations include invasive insects such as *Schistocerca nitens* (Thunberg) (= *S. vaga* Scudder), a grasshopper that consumes leaf, inflorescence, and fruit tissues (Alex Wegmann, U.S.F.W.S., pers. comm.); invasive plants; stochastic events; and/or reduced reproductive vigor (U.S.F.W.S. 1998).

Due to these threats, *P. remota* is currently listed as an endangered species. Recovery actions include developing long-term seed storage, propagation, and out-planting techniques (U.S.F.W.S. 1998). However, *P. remota* seeds are not presently stored in genebanks because "they do not remain viable in storage" (U.S.F.W.S. 1998). Yet, there are no studies that report the tolerance of *Pritchardia* species to storage conditions. The first step in verifying whether tissues can be stored in the dry (*e.g.* 0.02-0.05 g H_2O/g dry weight), cold (-18°C) conditions of genebanks is determining if they survive drying or reduced water potentials.

Assessing the desiccation tolerance of *Pritchardia* is hampered by their difficult physiology. Germination is delayed in seeds of *Pritchardia* species (Bornhorst 1996;

Culliney and Koebele 1999) and the mechanisms that control these delays are not understood. Clarifying these mechanisms can assist in forming propagation protocols for the creation of genetically diverse populations useful for recovery actions. Moreover, introduced vertebrate predators are known to damage seeds of *Pritchardia* (Arcand *et al.* 2003). The effects of seed predation can hamper *in situ* and *ex situ* recovery efforts. It is not known if *P. remota* seeds can tolerate damage and remain viable.

Therefore, in this dissertation I attempted to ascertain 1) if developmental patterns in embryos of *P. remota* resembled developmental patterns of species that can or can not tolerate storage under genebank conditions; 2) if embryos are tolerant of extreme drying stress; 3) what type(s) of dormancy mechanisms are responsible for delayed germination and factor(s) that affect these mechanisms; and 4) if seeds retain the ability to germinate after damage.

CHAPTER 2

THE PROTRACTED DEVELOPMENTAL PROGRAM IN EMBRYOS OF *PRITCHARDIA REMOTA* (KUNTZE) BECC. AND ITS DEVIATION FROM ORTHODOX AND RECALCITRANT DEVELOPMENTAL PATTERNS

Introduction

This study was established to determine if embryo development of *Pritchardia remota* (Kuntze) Becc., an endangered palm endemic to Hawaii, resembles the developmental patterns reported for species with orthodox or recalcitrant physiology. Orthodox seeds undergo substantial drying as the final stage of development (Berjak *et al.* 1990; Berjak and Pammenter 1997; Kermode and Finch-Savage 2002) and can survive for long periods in post-harvest storage using conventional protocols of low moisture content and temperature (*i.e.*, $\leq$ 2-5%, -18°C) (Roberts 1973; Hong *et al.* 1998).

Alternatively, recalcitrant seeds are sensitive to post-harvest desiccation and chilling stress and they cannot be stored using conventional protocols (Roberts 1973; Black and Pritchard 2002). However, the recalcitrant classification appears as a continuum when desiccation and chilling sensitivity of diverse species is analyzed (Farrant *et al.* 1988; Berjak *et al.* 1990; Berjak and Pammenter 1997). Ellis *et al.* (1990; 1991) also identified seeds with a storage physiology they believed to be intermediate to orthodox and recalcitrant classifications. Seeds with intermediate storage physiology appear to tolerate drying to water potentials of -90 to -250 MPa. However, viability is substantially reduced with further drying and storage at -20° to 0°C. Recalcitrance poses a major obstacle for the *ex situ* conservation of plant genetic diversity. *Pritchardia* species are not currently held in genebanks because "they do not store well" (U.S.F.W.S.

1998) and are presumably recalcitrant. However, there are no studies that report the tolerance of *Pritchardia* species to storage conditions.

Seed Development in Palms is Prolonged

The fruiting phenology of many palms is characterized by prolonged development on the parent tree. Flowering typically coincides with the wet season and the time taken to develop fruits can be from 2-10 months (De Steven *et al.* 1987; Peres 1994). Mature fruits have been observed after 4 to >12 months of development for several Panamanian palms (De Steven *et al.* 1987) and 8 to 12 months of development for nine species of Brazilian palms native to *terra firme* forests (Peres 1994). Seed development in the African oil palm (*Elaeis guineensis* Jacq.) is complete at close to 7 months after flowering (Aberlenc-Bertossi *et al.* 2003). Fruit maturation for *Pritchardia hillebrandii* (Kuntze) Becc. and *Pritchardia kaalae* Rock lasts more than 12 months (Pérez, unpublished data).

In contrast, mature seeds are produced about 4 to 5 months after flowering for temperate forest trees such as *Aesculus hippocastanum* L. (Hippocastanaceae) and *Quercus robur* L. (Fagaceae) (Grange and Finch-Savage 1992; Farrant and Walters 1998). Fruit maturation in the tropical mangrove species *Avicennia marina* (Forsk.) Vierh (Avicenniaceae), and *Sonneratia alba* J. Smith (Lythraceae) is complete in less than two months (Coupland *et al.* 2005). *Shorea robusta* Gaertner f. (Dipterocarpaceae), a tree of tropical distribution, produces mature fruits 3- to 4-months after flowering (Singh and Kushwaha 2006). However, maturation can last 5 to 12 months in other tropical trees (Grange and Finch-Savage 1992; Farrant and Walters 1998; Schmidt 2000).

The duration of seed development in a variety of *Coffea* L. (Rubiaceae) species ranges from approximately 2- to 10.5-months (Walters 1998).

The Assumption of Recalcitrance in Palm Seeds

Geographical and morphological characteristics have been used in an attempt to differentiate species with orthodox and recalcitrant storage physiologies (Farrant *et al.* 1988; von Teichman and van Wyk 1991, 1994; Hong and Ellis 1996, 1998; Schmidt 2000; Dickie and Pritchard 2002). Perhaps due to a primarily tropical distribution, large seed size, and supposed short longevity (Corner 1966; Uhl and Dransfield 1987), many palm species are assumed to be recalcitrant. This assumption may be further complicated by conflicting reports of multiple storage classifications for single species. For example, *E. guineensis* is reported to be recalcitrant (Roberts 1973), orthodox (Grout *et al.* 1983), and intermediate (Ellis *et al.* 1991). The prevalence of studies that report the lack of desiccation or cold tolerance in tropical palm seeds (Becwar *et al.* 1983; Broschat and Donselman 1986; de Carvalho *et al.* 1988; Dickie *et al.* 1992; Andrade 2001; Martins *et al.* 2003; Raja *et al.* 2003; Bovi *et al.* 2004; Martins *et al.* 2004; Panza *et al.* 2004; Stringheta *et al.* 2004) may also contribute to the idea of widespread recalcitrance in seeds of Arecaceae. Species such as *Coccothrinax argentata* (Jacq). Bailey, *Phoneix dactylifera* L. (but see von Fintel *et al.* 2004), and *Sabal mexicana* Mart. are considered to be orthodox, while *Acoelorraphe wrightii* Grisebach & Wendland, Roystonea regia (Kunth) Cook, and *Thrinax morrisii* H. Wendl. are listed as possessing intermediate storage physiologies (Flynn *et al.* 2004).

However, recent evidence suggests that members of Arecaceae possess a wide range of storage physiologies and storage classifications must be assessed on a case-by-

case basis (Davies and Pritchard 1998; Dickie and Pritchard 2002; Aberlenc-Bertossi *et al.* 2003; Pritchard *et al.* 2004; von Fintel *et al.* 2004). Developmental studies are used to understand the basis of storage physiology for a species and the differential responses to desiccation of various recalcitrant and orthodox seeds (Farrant and Walters 1998). More research is needed regarding the *ex situ* conservation potential of this large (*ca.* 2700 spp.), ecologically, and economically important family, of which many members are endangered or threatened. Currently, storage and germination characteristics are known for only about 4-7% of palm species (Davies and Pritchard 1998; Wood and Pritchard 2003).

To monitor changes during development, fruits of *P. remota* were harvested at approximately 30-day intervals from inflorescences tagged at anthesis and changes in fresh and dry mass and water relations were recorded. Subsequently, changes in ultrastructure and sugar composition of developing embryos were determined. These characteristics were compared to levels of maturation drying, the water potential (Ψ_w) at shedding, reductions in vacuolar volume, differentiation of organelles, and concentration of soluble carbohydrate of some orthodox and recalcitrant species reported in the literature.

Materials and Methods

Plant Material

Pritchardia remota is a single-trunked palm about 4-5 m tall with unarmed petioles, costa-palmate leaves, and paniculate inflorescences. This species naturally occurs in two populations on Nihoa (23°3'N, 161°55'W), a small, uninhabited island in the northwest Hawaiian island chain, approximately 450 km from Honolulu, HI

(21°18'N, 157°49'W). The mating-system and pollination biology of *Pritchardia* species has not been investigated. Fruits are single-seeded drupes (Fig. 2.1) and the endospermic seeds possess a relatively thick coat. The embryo comprises less than 1% of total seed volume (Pérez unpublished data).

Accessible inflorescences from one tree, growing on the grounds of the University of Hawaii at Manoa (Honolulu, Oahu), were tagged at anthesis and fruits harvested at regular intervals throughout development. Flowering commenced in early-March, 2004, peaked in June, and ended in mid-August. Fruit shedding began in late-March, 2005 and continued sporadically until early-September. Fruits of different maturity classes, based on pericarp color, were also collected from three additional trees growing in Waimanalo, Oahu in 2005. These fruits were transported to the National Center for Genetic Resources Preservation (Ft. Collins, CO) by expedited air-courier within two days of collection and stored under laboratory conditions for no more than two weeks.

Water Content Determinations

Embryos were excised according to Pérez (2005) and the fresh mass of five embryos at each harvest date was recorded. Embryos were dried at 95°C for 96 h then dry mass was determined gravimetrically. Water content was calculated from embryo fresh and dry mass and is stated as g H_2O per g dry mass (g g^{-1}). The water content of pericarps and seeds without embryos was calcualted using the same procedures.

Water Potential Determinations

At each harvest date five embryos were immersed, individually, in polyethylene glycol (PEG, M_r = 8000) solutions of different concentrations (Ψ_w, -12 to -0.05 MPa) for 48 h at 25°C. The embryos were blotted dry after soaking, and then the fresh and dry

mass of each was recorded. Water content was determined as described above. Water potential of different PEG solutions was calculated according to Michel and Kaufman (1973) and verified by thermocouple psychrometry (Model SC-10, Decagon Devices, Pullman, WA, USA). Embryos were also dried over saturated solutions of KNO_3 (91% RH, Ψ_w = -12.9MPa); KCl (85% RH, Ψ_w = -22.3MPa); NaCl (75% RH, Ψ_w = -39.5MPa); $MgCl_2$ (32% RH, Ψ_w = -154.3MPa); LiCl (13% RH, Ψ_w = -280.1MPa); and P_2O_5 (0.3% RH, Ψ_w = -797.5MPa) for 14d at 25°C. Water content and water potential of developing embryos was related by constructing water sorption isotherms for PEG and saturated salt solutions.

Ultrastructure Studies

Five to 10 developing embryos were fixed in 4% glutaraldehyde and 2% paraformaldehyde in 0.05 M NaCacodylate buffer (pH = 7.2) overnight at 4°C. Embryos were post-fixed in 1% OsO_4 for 1 h, followed by dehydration in a graded ethanol-propylene oxide series. Following dehydration, embryos were infiltrated with dilutions of Spurr's resin and 100% propylene oxide. Embryos were then embedded in Sprurr's resin. Embryos were sectioned with a Reichert ultracut-E ultramicrotome (Wien, Austria). Sections were mounted on grids and stained with uranyl acetate followed by lead citrate. The sections were viewed with a LEO 912 transmission electron microscope, fitted with an Omega energy filter, at 100 kV.

Sugar Analyses

The type and amount of soluble sugars in developing embryos was determined using HPLC. Embryos previously frozen in liquid nitrogen and stored at -80 °C were lyophilized. For each developmental stage, 15-30 mg of freeze-dried material was

ground in a small amount of liquid nitrogen. Soluble sugars were extracted with 80% ethanol at 90°C for 20 min from three replicates of 5-10 mg each. The extraction was performed three times for each replicate. Samples were centrifuged at 2000 rpm for 2 min after extraction. Supernatants from each sample were pooled and dried under nitrogen gas. After drying, samples were stored at -80 °C until analysis.

Samples were re-suspended in 5 ml of de-ionized water, agitated on a vortex mixer, and held at room temperature for 30 min. Approximately 2 ml of sample was then filtered (0.20 μm syringe filter) into an auto-sampler vial. Separations were performed using three columns in sequence: a Dionex (Sunnyvale, CA) Borate Trap Column (4 x 80mm), a Dionex CarboPac PA10 guard column (4 x 80mm), and a Dionex CarboPac PA10 analytical column (4 x 250 mm). The following gradient program and eluants were used (E1 = deionized water; E2 = 500 mM NaOH; t = time in min): t = 0, 3.2% E2; t = 40 min, 3.2% E2; t = 45 min, 18% E2; t = 75 min, 18% E2; t = 75.1 min, 100% E2; t = 85 min, 100% E2; and t = 85.1 min, 3.2% E2. Injections were made every 95 min. Pulsed amperometric detection was used to identify sugars. Sugar standards were arabinose, fructose, fucose, galactose, glucose, maltose, sucrose, trehalose, raffinose, stachyose, and several sugar alcohols.

Results

P. remota Embryos Undergo Substantial Maturation Drying

Water content decreased early in development and coincided with an increase in dry matter accumulation and increases in fresh mass (Fig 2.2). Dry matter was accumulated progressively from about 100 to 230 days after flowering (DAF). Maximum dry weight was achieved between 230 to 250 DAF and remained steady, for

about 157 additional days, until shedding at 397 DAF (Fig. 2). The attainment of maximum dry weight suggests that embryos have reached 'physiological' (TeKrony and Egli 1995) or 'mass' (Ellis and Pieto-Filho 1992) maturity.

Water content in immature *P. remota* embryos was high (10.74 g g^{-1}, 70 DAF), but decreased to 1.73 g g^{-1} 340 DAF. From 340 DAF until shedding there were large decreases in fresh mass and water content of pericarps, seeds, and embryos (Fig. 2.2). Water content in embryos decreased to 0.43 g g^{-1} between 340 and 397 DAF. This change marks the stage called maturation drying. Similar changes were observed in pericarps and seeds (data not shown).

Embryonic Water Potential Drops Below –15 MPa *In Planta*

Water sorption isotherms show that water potential was also high early in development and decreased in three distinct steps during development (Fig 2.3). In the early stages before mass maturity (*e.g.* 70 to 190 DAF) water potential decreased from -0.01 to -0.18 MPa, along with a related reduction in water content. Even though water content of embryos at 135 DAF was consistent with similar days after flowering, water potential at this harvest date was -0.68 MPa. Fruits of this harvest were transported to Ft. Collins for measurement and differences may be due to the much drier conditions in Ft. Collins, CO compared to Honolulu, HI. Water potentials declined from -0.31 to -2.77 MPa during reserve accumulation (200 to 340 DAF). This reduction in water potential is in contrast to smaller changes in water content over the same period. The final step occurred during maturation drying. In this stage, water potential dropped from -2.77 MPa at 340 DAF to -59.26 MPa at 397 DAF.

Figures 2.4 and 2.5 show the water potential isotherms for developing embryos dried in PEG or over saturated salt solutions, respectively. Note that in Fig. 2.4 isotherms were calculated for embryos harvested at 135, 250, 370, and 397 DAF; while in Fig. 2.5 isotherms are for embryos harvested at 240, 280, and 397 DAF. In both cases, water potential is highest for the most immature embryos at the same water content. This result has been reported in muskmelon seeds (Welbaum and Bradford 1988), *Quercus robur* embryos (Grange and Finch-Savage 1992), and *Zizania* embryos (Vertucci *et al.* 1994).

Vacuolar Volume is Reduced and Organelles De-differentiated During Maturation

Figure 2.6 shows the ultrastructure of cells from embryos at 100 DAF. At this stage, cells are dominated by a large nucleus and a few large vacuoles. The large vacuoles are reduced in size through endo-membrane development. Some of the vacuoles contain electron-dense substances. Storage vesicles also form through endocytosis. At 250 DAF, cells have increased in size in order to accommodate accumulated reserve materials (Fig. 2.7). By 280 DAF synthesis of reserve materials, characterized by lipid droplets, is complete and the entire cellular volume appears filled (Fig. 2.8). This pattern is observed until shedding (Fig. 2.9, 2.10). At 397 DAF mitochondria were de-differentiated. However, the structural integrity of other organelles appeared to be maintained (Figs. 2.9 and 2.10).

Sucrose is the Predominant Sugar and Small Concentrations of Trehalose were Detected During Development

Soluble sugars and sugar alcohols were detected by HPLC during the developmental program of *P. remota* embryos. Sucrose was the major sugar found in developing embryos and accounted for 99.4 ± 0.09% (mean ± SE) of the total soluble

sugar fraction (Table 2.1). The sucrose content remained high during reserve accumulation but fell with the onset of maturation drying

Trehalose, considered to occur rarely in angiosperms, was detected sporadically and in small concentrations during development. Trehalose content peaked approximately two months before the start of maturation drying but was still evident at the onset of maturation drying. However, no trehalose was found in embryos at shedding.

Total oligosaccharide content (raffinose + stachyose) was low compared to other types of soluble sugars and sugar alcohols. Furthermore, total oligosaccharide content varied in development. The oligosaccharide to sucrose ratio (O:S) decreased during development, but eventually increased at shedding (Table 2.1).

Fructose and glucose dominated the total monosaccharide content of developing embryos (Table 2.1). However, fructose was not detected until shedding. On the other hand, glucose was found consistently until maturation drying, but was not detected at shedding. Total monosaccharide content decreased throughout development and the monosaccharide to sucrose ratio (M:S) at shedding was 0.0021.

Myo-inositol, arabitol, and sorbitol were consistently observed throughout the developmental program. Total sugar alcohol content was higher than total mono- and oligosaccharide contents, with some exceptions, but lower than total disaccharide content. Total sugar alcohols decreased during development.

Discussion

Developmental patterns displayed by *P. remota* indicate that seed tissues are neither recalcitrant nor orthodox. Rather, they have characteristics of each type. Post-

histodifferentiation developmental patterns provide the most compelling differences between orthodox and recalcitrant species. In terms of water content, recalcitrant seeds remain hydrated throughout development (Finch-Savage 1992; Tompsett and Pritchard 1993; Farrant *et al.* 1997; Kermode and Finch-Savage 2002). Although many recalcitrant seeds experience some reductions in water content during development, as a consequence of dry matter accumulation, the decline is not as striking as the moisture loss in orthodox seeds during maturation drying (Hong and Ellis 1990; Farrant *et al.* 1997). At shedding, embryos of *P. remota* have a water content of 0.43 g g^{-1} ± 0.06 (mean ± SE). This is similar to the shedding water content of recalcitrant *Theobroma cacao* L. embryos (Alemanno *et al.* 1997) and embryonic axes of some *Inga* Willd. species (Pritchard *et al.* 1995). In contrast, oil palm (Aberlenc-Bertossi *et al.* 2003) and *Phoenix reclinata* Jacquin. (von Fintel *et al.* 2004) embryos had water contents of 2.0 and 1.5 g g^{-1} at shedding, respectively. Furthermore, embryonic water potential drops to about -60 MPa at shedding for *P. remota*. This value is well below the lower limit reported for recalcitrant embryos (Pritchard 1991; Finch-Savage *et al.* 1992; Grange and Finch-Savage 1992; Vertucci and Farrant 1995; Sun and Liang 2001; Walters *et al.* 2001; Black and Pritchard 2002; Pritchard 2004).

Orthodox seeds are said to reach 'physiological' or 'mass' maturity because they attain a maximum level of dry weight before shedding (Ellis and Pieto-Filho 1992; TeKrony and Egli 1995; Kermode and Finch-Savage 2002). On the other hand, recalcitrant seeds may continue to accumulate dry matter until shedding, suggesting no clear end point to development (Farrant *et al.* 1988; Berjak *et al.* 1990; Finch-Savage *et al.* 1992; Tompsett and Pritchard 1993; Berjak and Pammenter 1997; Berjak and

Pammenter 2000; Berjak 2005). *Pritchardia remota* embryos attain maximum dry weight between 230 to 250 DAF and therefore are more like orthodox seeds than recalcitrant seeds in this regard.

Desiccation sensitive cells often have extensive vacuolation, lack of reserve accumulation, and differentiated organelles (*i.e.* metabolically active) (Poulsen and Eriksen 1992; Finch-Savage and Blake 1994; Vertucci and Farrant 1995; Farrant and Walters 1998; Pammenter and Berjak 1999; Berjak 2005). *Avicennia marina*, a highly recalcitrant species, has seeds that display all three characteristics. However, moderately recalcitrant seeds such as *A. hippocastanum* show limited vacuolation and accumulation of reserves, but organelles remain in an apparently active state (Vertucci and Farrant 1995; Farrant *et al.* 1997; Farrant and Walters 1998). Comparatively, the orthodox seeds of *Phaseolus vulgaris* L. have decreased vacuolation, increased accumulation of insoluble reserves, and de-differentiation of organelles (Farrant *et al.* 1997).

The studies presented here suggest that *P. remota* embryos share ultrastructural characteristics with moderately recalcitrant and/or orthodox species, but not highly desiccation sensitive tissues. Similarly, mature embryos of *P. reclinata* presented organelles with limited de-differentiation at shedding and are considered to possess intermediate storage physiology (von Fintel *et al.* 2004). In contrast, embryos of the recalcitrant palm *Euterpe edulis* Martius had well-developed mitochondrial cristae and a high degree of vacuolation at shedding (Panza *et al.* 2004).

The accumulation of soluble carbohydrates, especially non-reducing disaccharides and oligosaccharides, has been implicated as a mechanism that confers desiccation tolerance among orthodox species (Hoekstra and Van Roekel 1988; Koster and Leopold

1988; Chen and Burris 1990; Blackman *et al.* 1992; Corbineau *et al.* 2000; Bailly *et al.* 2001). However, using the quantity or quality of soluble carbohydrates to determine whether a species can tolerate desiccation is confounded by the fact that recalcitrant seeds accumulate the same types and amounts of soluble carbohydrates as orthodox seeds (Pritchard *et al.* 1995; Alemanno *et al.* 1997; Li and Sun 1999; Bucheli *et al.* 2001). For instance, in one study, embryos of *Cocos nucifera* L. (recalcitrant) and *E. guineensis* (intermediate) had the highest total sugar contents (*e.g.*, 296.4 ± 52.6 and 215.9 ± 21.2 mg g^{-1} dw respectively, mean ± SE) of all species tested (Steadman *et al.* 1996). Embryos of two sympatric *Acer* (Aceraceae) species accumulated sucrose, raffinose, and stachyose during development. However, *Acer platanoides* L. (orthodox) had slightly higher contents of these sugars than *Acer pseudoplatanus* L. (recalcitrant) and this accounted for the greater desiccation tolerance in the former (Greggains *et al.* 2000). Moreover, no correlation was found between total soluble carbohydrate, oligosaccharide content, sucrose content and storage longevity for 276 species (Walters *et al.* 2005).

Nonetheless, the ratio of oligosaccharides to sucrose (O:S) may be useful to initially screen species in terms of storage physiology (Lin and Huang 1994; Steadman *et al.* 1996). Steadman *et al.*, (1996) found that O:S ratios between desiccation tolerant and sensitive species differed significantly. Orthodox species generally had a O:S ratio greater than 0.143, while the O:S ratio for recalcitrant seeds was less than 0.083. Yet, the O:S ratio was not found to be a reliable indicator to distinguish between recalcitrant or intermediate species within a genus (Chabrillange *et al.* 2000). Desiccation tolerance has also been associated with an decrease in monosaccharide contents and M:S ratios during development. A reduced level of monosaccharides may reduce the level of substrates

available for deleterious metabolic reactions (Leprince *et al.* 1990; Leprince *et al.* 1992). In *P. remota*, the total monosaccharide and oligosaccharide pools were small throughout development. Total monosaccharides decreased as development progressed.

Despite the low levels of mono- and oligosaccharides, some interesting trends emerged during development. For example, sucrose was found in remarkably high concentrations. This is not surprising however. *Cocos nucifera* and *E. guineensis* embryos had the highest sucrose contents (*e.g.*, > 200 mg g^{-1} DW) of any species in the Steadman *et al.* (1996) study. It is interesting that small amounts of trehalose were detected. Also some raffinose and stachyose were detected before seeds were shed. This suggests that sucrose, in conjunction with trehalose and the oligosaccharides, may be one mechanism conferring tolerance to low water potentials during development. Additionally, small amounts of sugar alcohols were detected in developing *P. remota* embryos and may be related to increased stress tolerance (Buitink *et al.* 2002).

After an initial decrease, the O:S ratio in *P. remota* embryos remained steady for the bulk of development then increased to 0.0026. Similarly, the O:S ratio increased during development of *E. guineensis* (Aberlenc-Bertossi *et al.* 2003). Although an increase in O:S is associated with desiccation tolerance, the value calculated for *P. remota* is lower than for other non-orthodox palms. Using data in Steadman *et al.* (1996), mature embryos of *C. nucifera* and *E. guineensis* had O:S values of 0.03 and 0.25, respectively. Similarly, the O:S ratio for mature *E. guineensis* embryos was 0.20 (Aberlenc-Bertossi *et al.* 2003). The O:S and M:S may not be good indicators of desiccation tolerance in *P. remota*.

The evidence presented in this study suggests that developmental patterns in *P. remota* are intermediate to those observed for recalcitrant and orthodox species. However, viability of intermediate tissues is known to be of limited duration under conventional genebank conditions (Ellis *et al.* 1990, 1991; Walters *et al.* 2002; Pritchard 2004). It may be necessary to employ other techniques, such as cryo-preservation, to extend the longevity of this species in storage. This dichotomy highlights the need for further research into the developmental physiology of palm seeds.

Tables

Table 2.1. Concentration of soluble sugars and sugar alcohols identified by HPLC in developing *Pritchardia remota* embryos. M:S and O:S are monosaccharide to sucrose and oligosaccharide to sucrose ratios.

	mg/g DW Days After Flowering					
	160	190	250	280	340	397
Alcohols						
myo-inositol	1.19	1.19	0.74	1.21	1.03	1.09
arabitol	0.40	0.30	0.28	0.45	0.42	0.45
sorbitol	0.82	0.71	1.26	0.35	0.27	0.00
Total	2.41	2.20	2.28	2.01	1.72	1.54
Monosaccharides						
arabinose	0.57	0.05	0.00	0.00	0.22	0.04
fructose	0.00	0.00	0.00	0.00	0.00	0.66
fucose	0.11	0.00	0.00	0.07	0.05	0.06
galactose	0.11	0.15	0.23	0.19	0.00	0.00
glucose	0.86	1.07	1.06	0.89	0.79	0.00
Total	1.65	1.27	1.29	1.15	1.06	0.76
Disaccharides						
Reducing						
maltose	0.71	0.00	0.00	0.00	0.34	0.34
Non-reducing						
sucrose	437.97	422.31	434.61	418.76	363.09	369.96
trehalose	0.98	0.00	0.59	0.96	0.73	0.00
Total	439.66	422.31	435.20	419.35	364.16	370.30
Oligosaccharides						
raffinose	0.82	0.00	0.00	0.00	0.00	0.62
stachyose	0.38	0.45	0.40	0.26	0.18	0.35
Total	1.20	0.45	0.40	0.26	0.18	0.97
M:S	0.0038	0.0030	0.0030	0.0028	0.0029	0.0021
O:S	0.0027	0.0011	0.0009	0.0006	0.0005	0.0026

Figures

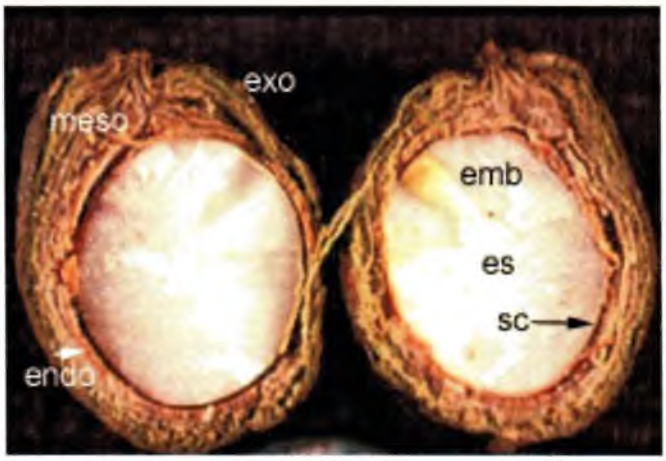

Fig. 2.1. Cross section of *Pritchardia remota* fruit and seed. Exo = exocarp, meso = mesocarp, Endo = endocarp, SC = seed coat, ES = Endosperm, Emb = embryo.

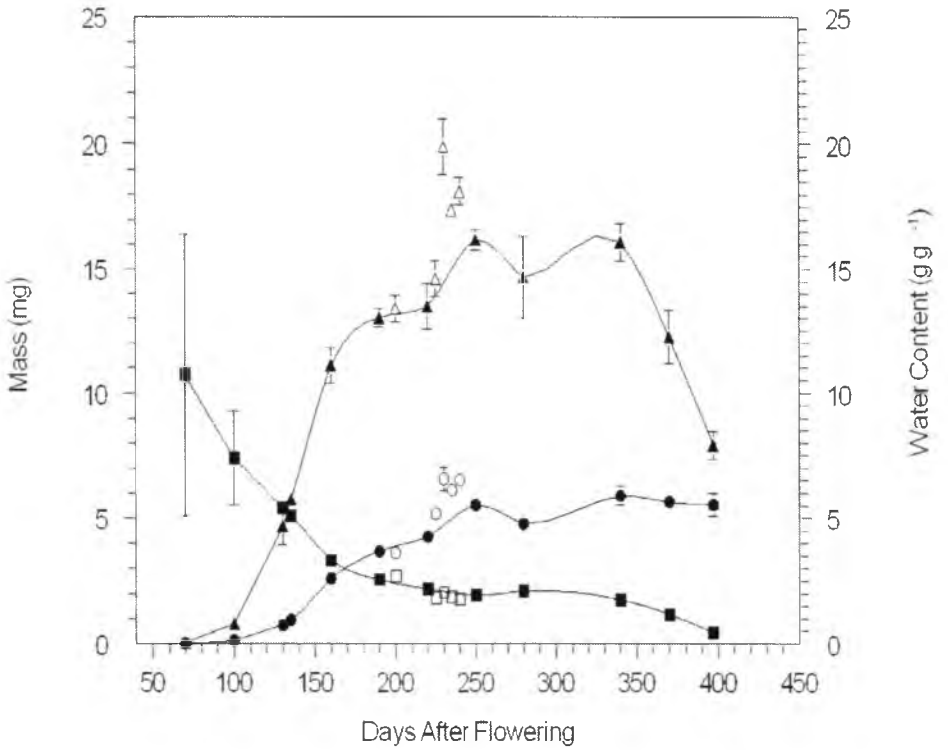

Fig. 2.2. Changes in fresh (triangles) and dry (circles) mass and water content (squares) of developing *Pritchardia remota* embryos collected in 2004-05 from the University of Hawaii at Manoa (closed symbols) or Waimanalo, Oahu (open symbols). Data are means of five embryos ± SE.

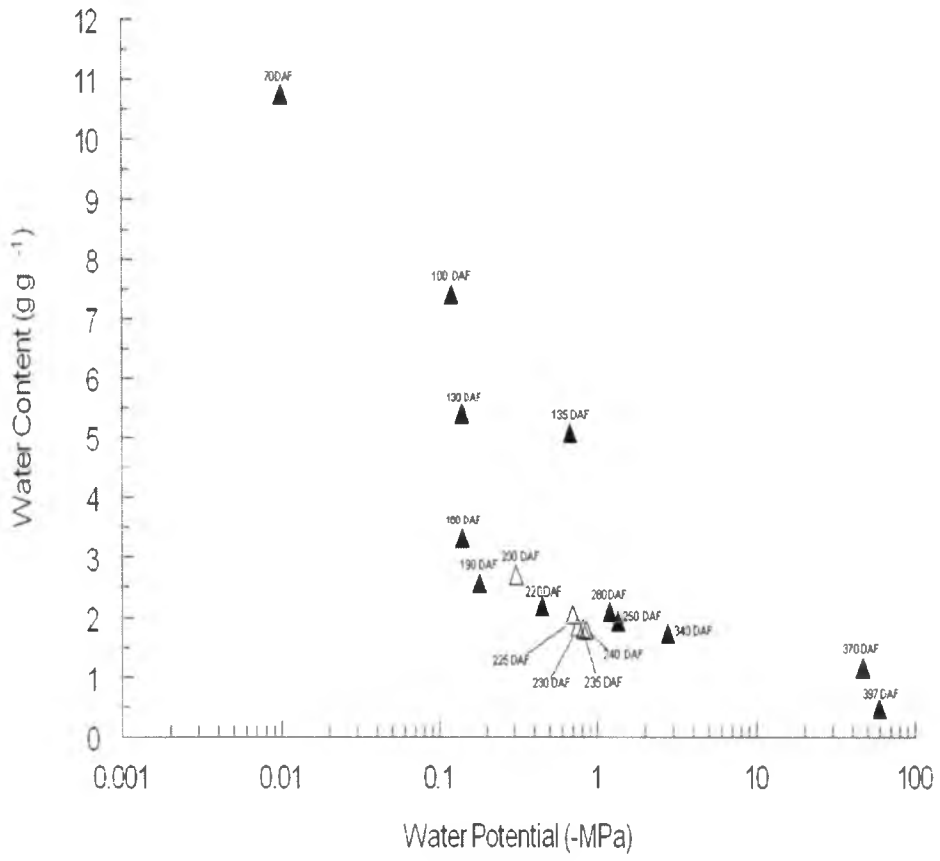

Fig. 2.3. The relationship between water content and water potential of developing *P. remota* embryos. Open and closed figures as in 2.2.

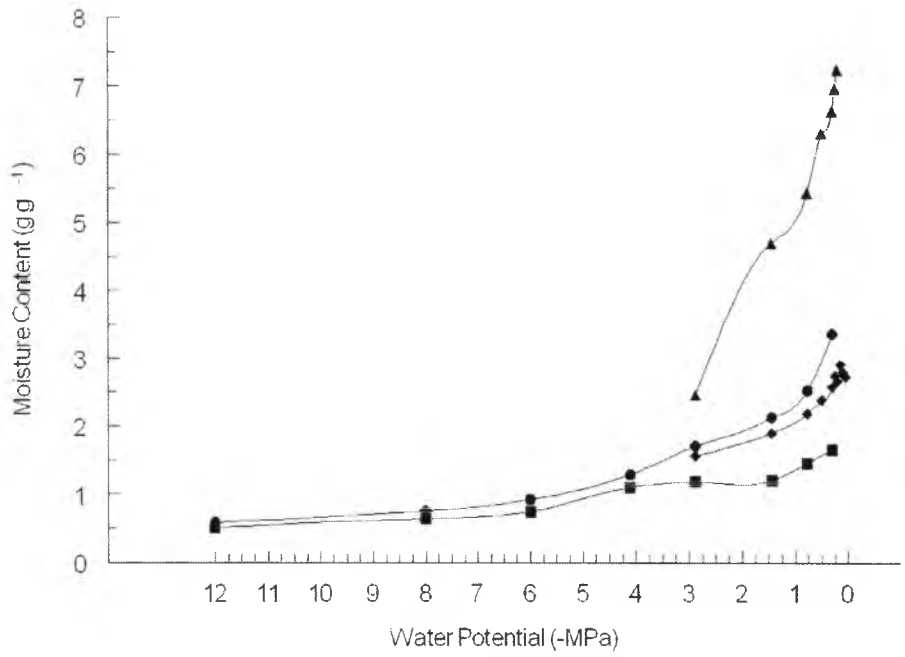

Fig. 2.4. Sorption isotherms for *Pritchardia remota* embryos, harvested at 135 (triangles), 250 (circles), 370 (diamonds), and 397 (squares) days after flowering, and imbibed in solutions of PEG for 24 h at 25°C.

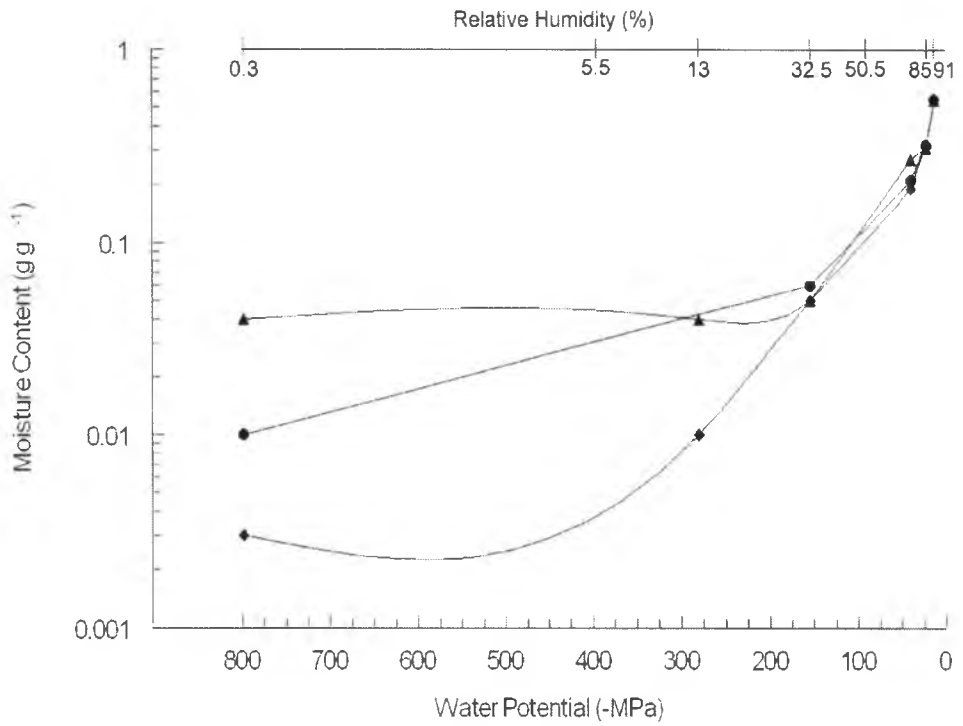

Fig. 2.5. Sorption isotherms for *Pritchardia remota* embryos, harvested at 240 (triangles), 280 (circles) and 397 (diamonds) days after flowering, and incubated at 25°C over various saturated salt solutions.

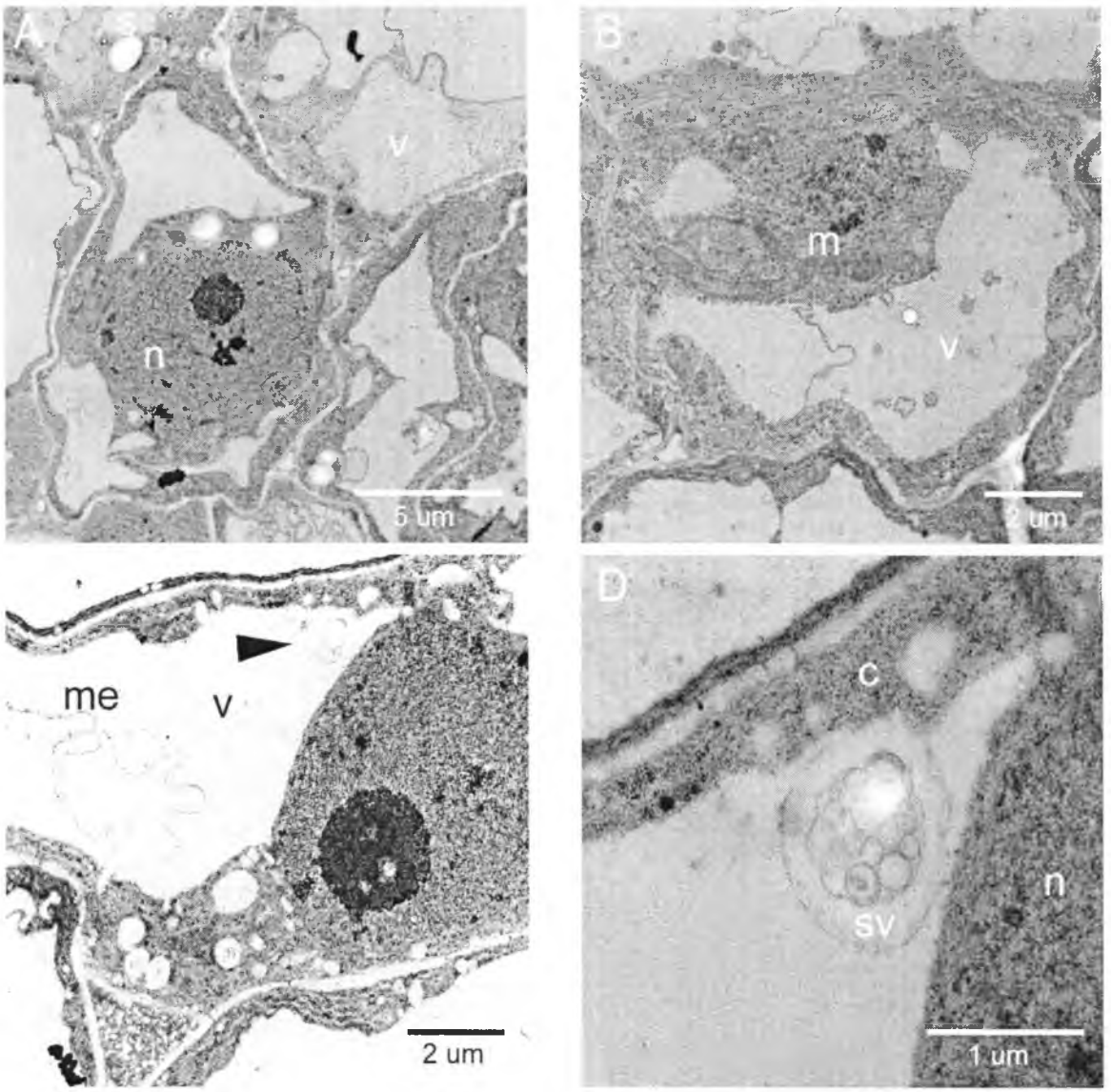

Fig. 2.6. Transmission electron micrographs of cells from 100 DAF embryos. Note the large nucleus and vacuoles being divided through endomembrane development (A, B, C). n = nucleus; v = vacuole; s = starch grain; m = mitochondria; me = endomembrane; arrow head and sv = storage vesicle; c = cytoplasm. (D) Formation of storage vesicle through endocytosis.

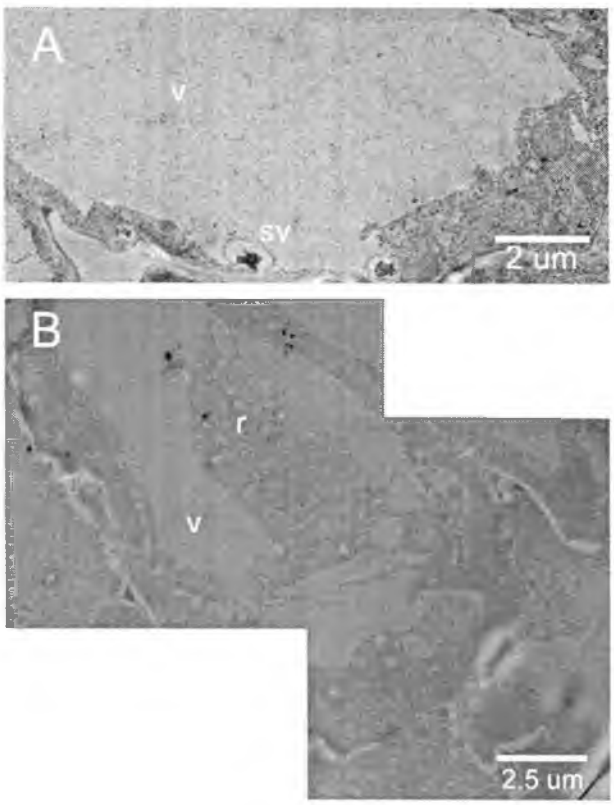

Fig. 2.7. Micrographs of *P. remota* embryo cells at 250 DAF. (A) Vacuole filled with ergastic substances. (B) Maximum dry weight has been achieved and reserve synthesis is underway. v = vacuole; sv = storage vesicle; r = synthesized reserve materials. The micrograph in (B) is a composite image.

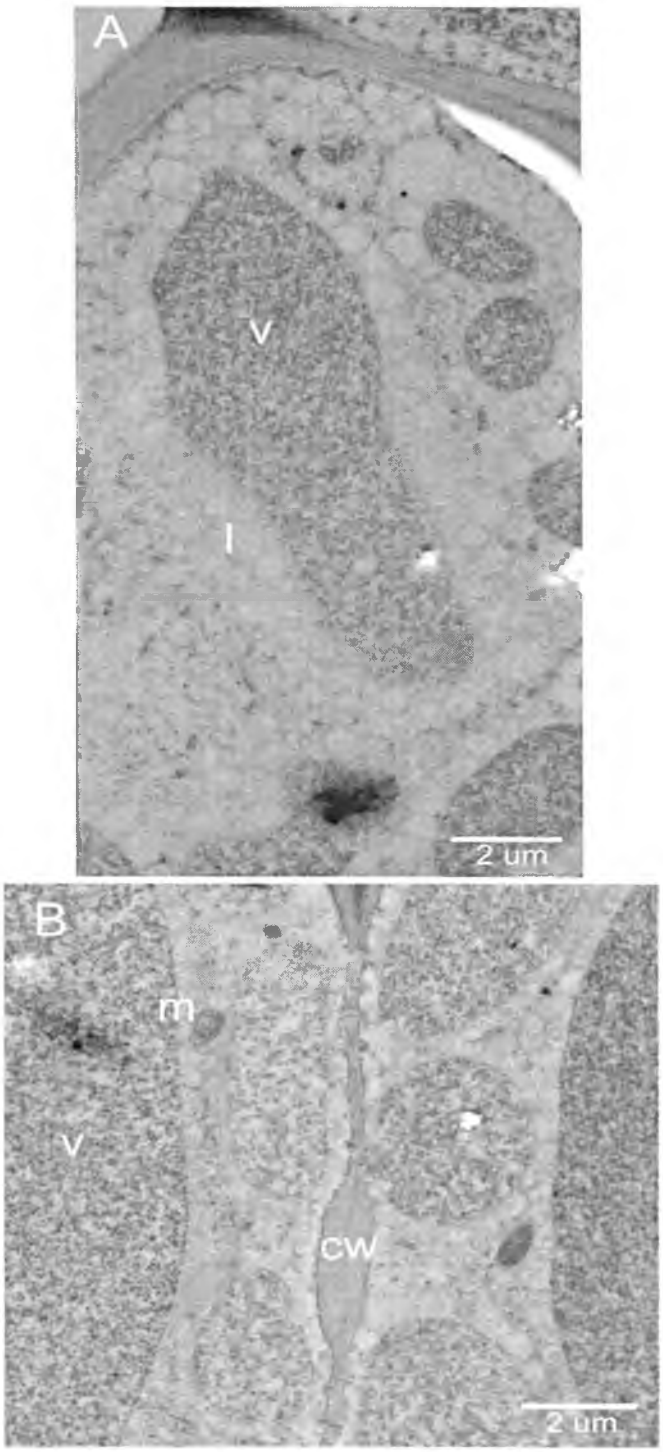

Fig. 2.8. Embryonic cells of *P. remota* at 280 DAF. (A) At this stage reserves have been completely synthesized and occupy the cell volume. (B) Two adjacent cells showing very few mitochondria. cw = cell wall; l = lipid droplet; m = mitochondria; v = vacuole.

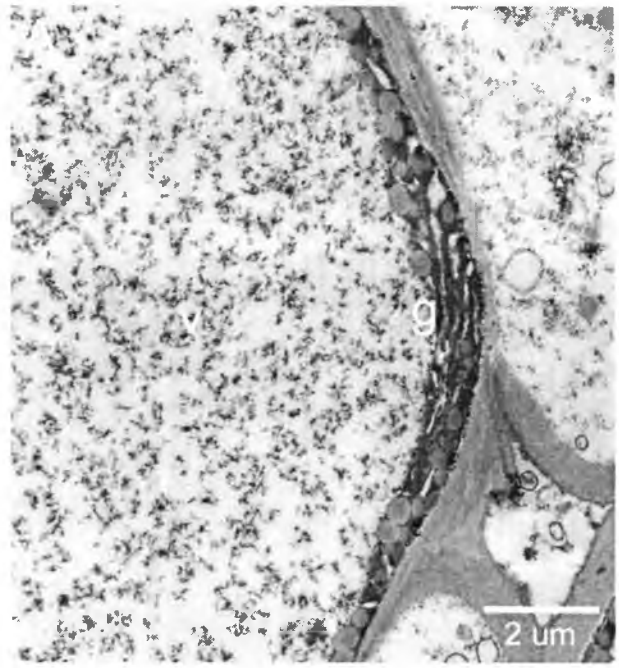

Fig. 2.9. Micrograph of cells at 340 DAF. The large vacuole (v) is filled with ergastic substances and has reduced the cytoplasmic volume. Golgi bodies (g) appear intact but constrained due to the vacuole.

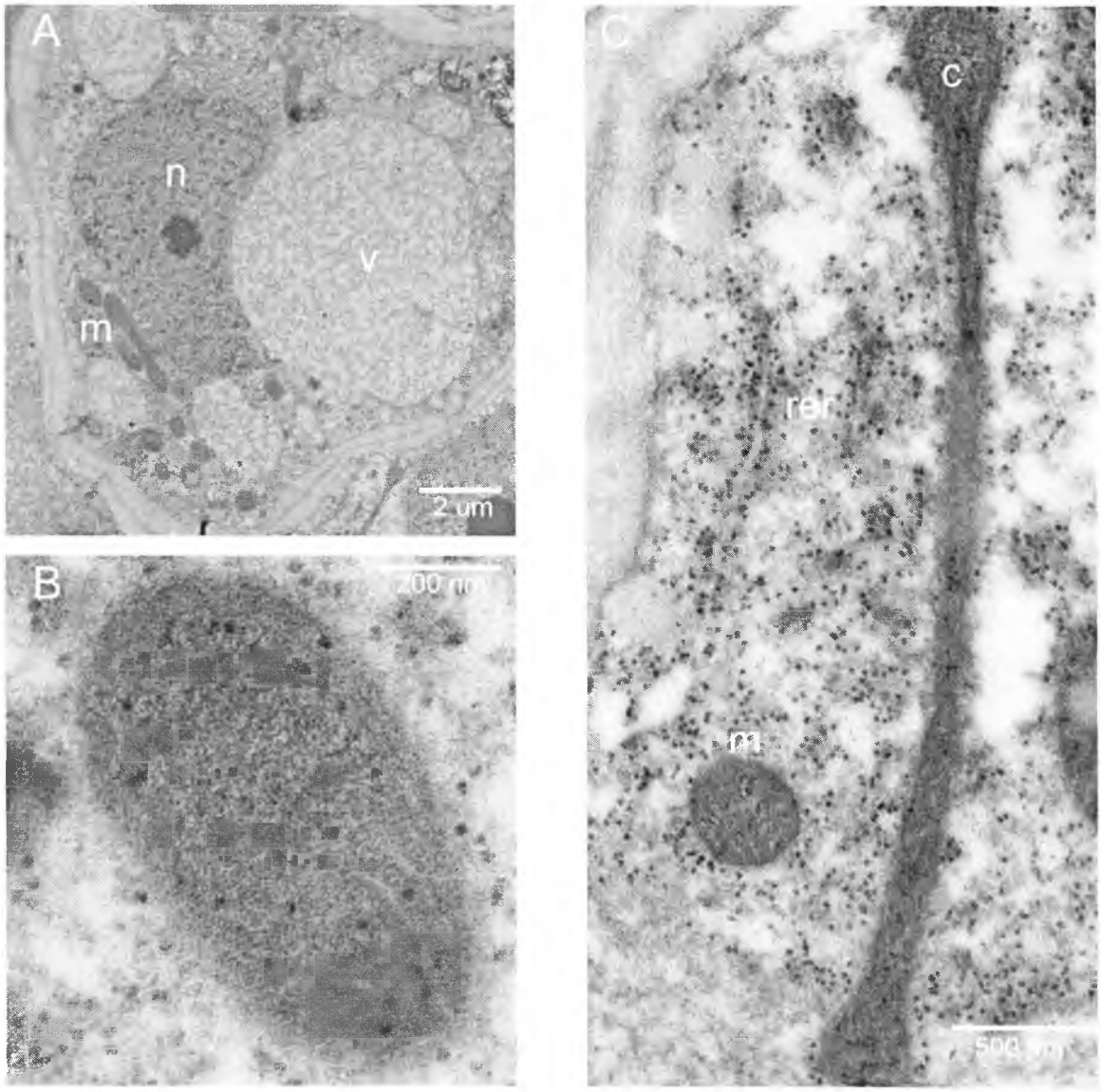

Fig. 2.10. *P. remota* cells from 397 DAF embryos. (A) Vacuoles packed with reserve materials and few mitochondria visible. (B) Detail of de-differentiated mitochondria. (C) De-differentiation of mitochondria (m) and rough endoplasmic reticula (rer). However, cisternae (c) of the Golgi complex appear intact.

CHAPTER 3

GERMINATION OF MATURING EMBRYOS OF *PRITCHARDIA REMOTA* (KUNTZE) BECC.: INDICATIONS FROM DEVELOPMENTAL AND DESICCATION STRESS EXPERIMENTS

Introduction

Perhaps because of their tropical distribution, large seed size, and presumed short lifespan (Corner 1966; Uhl and Dransfield 1987) members of Arecaceae (= Palmae) were assumed to produce seeds that do not survive storage using conventional genebank conditions (*i.e.*, -18°C, 2-5% moisture content). However, recent evidence suggests that seed storage physiology within Arecaceae is varied (Dickie *et al.* 1992; Dickie and Pritchard 2002; Flynn *et al.* 2004). For example, species such as *Coccothrinax argentata* (Jacq). Bailey, *Phoenix dactylifera* L. (but see von Fintel *et al.* 2004), and *Sabal mexicana* Mart. are considered orthodox (*i.e.* amenable to conventional storage conditions), while *Areca catechu* L., *Euterpe edulis* Mart., and *Mauritia flexuosa* L f. are recalcitrant. *Acoelorraphe wrightii* Grisebach & Wendland, *Roystonea regia* (Kunth) Cook, and *Thrinax morrisii* H. Wendl. are listed as possessing intermediate storage physiologies (Flynn *et al.* 2004).

The seed storage classification for endemic Hawaiian *Pritchardia* is uncertain (Flynn *et al.* 2004). Despite its endangered status, seeds of *Pritchardia remota* (Kuntze) Becc. are not held in genebanks because they are considered "not to store well" (U.S.F.W.S. 1998). Recent studies (see Chapter 2) suggest that *P. remota* embryos have developmental patterns that deviate from patterns observed for recalcitrant species (Farrant *et al.* 1986; Vertucci and Leopold 1987; Farrant *et al.* 1988; Berjak *et al.* 1990;

Vertucci and Roos 1990; Vertucci and Farrant 1995; Berjak and Pammenter 1997; Farrant *et al.* 1997; Farrant and Walters 1998). For example, *P. remota* embryos undergo substantial maturation drying. Water content at the very early stages of development (*cf.* 70 days after flowering) is about 11 g H_20 g^{-1} DW (g g^{-1}), but then decreases to 0.43 g g^{-1} at shedding. Furthermore, embryonic water potential declines to about -60 MPa at shedding, a value well below the -15 MPa desiccation tolerance limits of mature recalcitrant tissues (Vertucci and Farrant 1995; Black and Pritchard 2002; Walters *et al.* 2002; Walters 2004). The relatively low embryonic water potential is not detrimental as *P. remota* seeds germinate after dormancy is alleviated (see Chapter 4). Moreover, mitochondrial de-differentiation is observed in embryonic cells of *P. remota*. On the contrary, mitochondria of mature *E. edulis* (recalcitrant) embryos have well-developed cristae (Panza *et al.*, 2004).

Although *P. remota* embryos possess mechanisms to tolerate substantial water loss throughout development, the effect of post-harvest desiccation to enable storage under genebank condition is not clear. Here we address the hypotheses that 1) embryos do not increase in desiccation tolerance throughout development, 2) mature embryos do not exhibit the extreme levels of desiccation tolerance observed in tissues stored under conventional genebank conditions, and 3) metabolic activity of embryos is not suppressed in the developmental program. To test these hypotheses we dried developing and mature embryos to different water contents using a stream of nitrogen gas (N_2) or saturated salt solutions. We also measured oxygen (O_2) uptake rates and antioxidant activity in fresh or dried embryos, comparing this to orthodox and recalcitrant species.

Materials and Methods

Plant Material

Fruits of various developmental stages were collected as described in Chapter 2. Two additional batches of developing fruits were collected and transported, within two days of collection, to the National Center for Genetic Resources Preservation (Ft. Collins, CO) from Hawaii as airline carry-on baggage or by expedited air courier. Fruits from the second harvest were divided into different maturity classes based on pericarp color. Fruits from any harvest were stored under laboratory conditions for no more than two weeks and embryos extracted from seeds immediately preceding experimentation.

Embryo Germination in Tissue Culture

Ten embryos per harvest date were excised according to Pérez (2005) and surface sterilized for 5 min in a 10% commercial bleach solution containing two drops of Tween-20. Sterilized embryos were then cultured aseptically on solid, half-strength Murashige and Skoog basal medium with sucrose and agar (Sigma, St. Louis, MO); medium pH was adjusted to 5.7 with 1 N NaOH (Nellie Sugii, pers. comm.). Cultured embryos were kept at room temperature and under fluorescent lights. Embryos were classified as germinated after they had enlarged and produced radicles $\geq$ 5 mm.

Desiccation Tolerance of Developing Embryos

Embryos (n = 12) were 'flash' dried (Pammenter *et al.* 1991) over a stream of N_2 (500 ml/min) for 0 to 1200 min. Water content, on a dry weight basis, was determined for two embryos at each drying time. The remaining embryos were re-hydrated on moist blotter (5 ml H_20/blotter) in Petri dishes for 45 to 60 min. Two replicates of five re-hydrated embryos from each drying time, were then immersed in 5 ml of distilled, de-

ionized water and the electrical conductivity of the steep water was measured with a CDM 83 conductivity meter (Radiometer Copenhagen, Copenhagen, Denmark) every 10 min for 1 h. Following conductivity measurements, embryos were cultured as above.

Embryos were dried to low water content (< 0.10 g g^{-1}) using saturated salt solutions that controlled relative humidity. To determine how long embryos survived at low water potential (Ψ_w), embryos were placed in Petri dish bottoms within sealed desiccation jars and dried over saturated solutions of KNO_3 (RH = 91%, Ψ_w = -13 MPa), KCl (RH = 85%, Ψ_w = -22 MPa), NaCl (RH = 75%, Ψ_w = -39 MPa), $MgCl_2$ (RH = 33%, Ψ_w = -154 MPa), LiCl (RH = 13%, Ψ_w = -280 MPa), and P_2O_5 (RH = 0.3%, Ψ_w = -797 MPa) at 25°C for 3 to 43 d. Embryos held over KNO_3 and KCl were surface sterilized, transferred to the desiccation chambers under aseptic conditions, and fresh weights taken in a ventilated tissue culture hood to minimize contamination. However, some embryos became contaminated with fungi and were subsequently discarded. Embryos not visibly contaminated were handled as described previously for germination assays.

Oxygen Consumption by Developing Embryos

Oxygen consumption rates were used to estimate metabolic rates of developing *P. remota* embryos and embryos or axes of lychee (*Litchi chinensis* Sonn.), tea (*Camellia sinensis* (L.) O. Kuntze), and pea (*Pisum sativa* L.) immediately after excision, drying in PEG solutions (-2.89 to -0.33 MPa) for 24 h, or drying over a stream of N_2. Oxygen uptake was measured manometrically with a Gilson differential respirometer. This technique was used because the effects of different water contents could be measured during experimentation. To absorb CO_2, 1 ml of a 10% (w:v) KOH solution was injected into the central well of Warburg flasks and wicked with folded filter paper strips.

Measurement of *P. remota* oxygen uptake was taken on five embryos, replicated two to three times, at 25°C over 8 h. Oxygen uptake rates were calculated from the slopes ($r^2 \geq 0.90$) of pressure-time course data and adjusted for embryo dry weight (Walters *et al.* (2001).

Trolox Equivalent Antioxidant Capacity (TEAC) of Developing Embryos

The TEAC assay, a photometric technique, measures the ability of total antioxidants to reduce ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical cations compared to the antioxidant activity of Trolox®. Oxidized ABTS (blue-green color) is reduced by antioxidants and de-colorized. At each developmental stage, 75-100 mg of lyophilized material was ground to a fine powder in liquid nitrogen. Three replicates of 25 mg were weighed out and placed in reaction vials with 75% ethanol. The remaining material was divided into five replicates of approximately equal weight for moisture content determinations.

Hydrophilic antioxidants were extracted in 5 ml of 75% MeOH for 60 min at 30 °C, with stirring, under a gentle stream of N_2. All vials were centrifuged at 3500 rpm for 5 min and three replicates of supernatant were placed in 15 ml plastic tubes. The extraction was repeated for 15 min in 2 ml of 75% MeOH. Triplicate samples of the supernatant were pooled with supernatant from the previous extraction. MeOH was evaporated from supernatants under N_2 until the volume in tubes was ≤ 2 ml. Tubes with remaining extract were plunged into liquid nitrogen, lyophilized, and stored at –20°C until use.

To determine antioxidant capacity in the lipophilic extractions, 4 ml of acetone was added to the residue remaining in the reaction vials. All extraction conditions were

the same as above, except that the lipophilic extraction lasted 15 min. The extraction was repeated using 2 ml of acetone for 15 min. Supernatants from each extraction were pooled and dried completely under N_2. After drying, all samples were stored at -20 °C.

Trolox standard solution (0.5 mM) was prepared by dissolving 6.26 mg Trolox (Sigma-Aldrich) in 50 ml of 5 mM phosphate buffered saline (PBS, pH 7.4) and stored in 1 ml aliquots at –20°C until use. ABTS solution (5 mM) was freshly prepared for each assay by dissolving 2.74 mg ABTS per milliliter of de-ionized water (80 ml). Magnesium IV oxide (2 - 5 g) was added to the solution and stirred gently for 20 min, at which point the solution turned blue-green. The solution was then filtered (0.45 μm syringe filter). Trolox standards (3 reps each) were also freshly prepared by sonicating 1 ml of frozen Trolox solution and diluting with PBS. Final Trolox concentrations equaled 0, 5, 10, 15, and 20 μmol/L.

The spectrophotometer (Spectronic Helios β, Thermo-Spectronic, Cambridge, UK) was set to 734 nm and zeroed with 100 μl PBS in 1 ml of de-ionized water. The absorbance of ABTS solution was read at 734 nm and diluted with 5 mM PBS as necessary to attain an absorbance of 0.700. Hydrophilic samples were re-suspended in 10 ml of MeOH, while lipophilic samples were re-suspended in 2 ml acetone. Sample or standard (100 μl) solutions were added to 10 x 75 mm test tubes followed by 1 ml of 5 mM ABTS solution. All solutions were vortexed immediately for 2 sec. Solutions were poured into semi-micro spectrophotometer cuvettes. Absorbance was read 2 min after adding ABTS solution. To correct sample absorbance values for appropriate solvents three replicates (100 μl) of MeOH or acetone were also prepared and 1 ml of 5 mM ABTS was added. Samples were vortexed and absorbance determined as above.

Results

Excised Embryos Germinate Early in the Developmental Program

Embryos gained the ability to germinate in tissue culture early (*i.e.*, 130 DAF) in the developmental program (Figs 3.1 and 3.2). At this time water content is about 7.4 g g^{-1}, but decreasing (see Chapter 2). Embryos achieved the capacity for total germination 100 d before attainment of maximum dry weight (230 to 250 DAF) and maintained full germination until the onset of maturation drying (340 DAF). From maturation drying onward the ability of excised embryos to readily germinate diminished. At shedding, the final germination of embryos in tissue culture did not exceed 25%. This is in sharp contrast to the high germination (100%) observed for intact seeds at shedding after receiving dormancy-breaking treatments (see Chapter 4, note that seeds in the germination experiments came from the same trees used in the present study).

Critical Water Contents Decrease Throughout Development but Embryos are not Tolerant of Extreme Drying or Prolonged Storage at High Water Contents

Critical water content is defined as "the minimum water content that cells can be dried to without imposing irreparable damage" (Black and Pritchard 2002) and it corresponds to a critical water potential (Farrant and Walters 1998). Conductivity was not a good measure of damage in developing *P. remota* embryos. Therefore, the critical water content of each developmental stage was identified by the intersection of regression lines marking a significant decrease in germination of embryos in tissue culture (Fig. 3.3).

P. remota embryos are irreparably damaged at a relatively high water content and water potential early in the developmental program. However, these critical values

decreased markedly as development progressed (Figs 3.4 and 3.5). Critical water content decreased from 2.31 g g^{-1} at 100 DAF to 0.21 g g^{-1} at 230 DAF; the latter harvest date marking the achievement of maximum dry weight, or 'mass maturity' (Ellis and Pieto-Filho 1992), in embryos. The rate of decrease in critical water content slowed after embryos had reached their maximum dry weight. For example, the critical water content decreased from 0.21 to 0.16 g g^{-1} between 230 and 340 DAF (Fig. 3.4).

Tolerance of rapid drying to relatively low water contents occurred in conjunction with maximum dry weight. Further improvements in desiccation tolerance were observed with the onset of maturation drying. It was not possible to determine critical values for the final stage of development due to the inability of embryos to germinate in tissue culture. However, the critical water content and critical water potential of embryos at 340 DAF were 0.16 g g^{-1} and -48 MPa, respectively (Figs. 3.4 and 3.5).

Embryos that were flash dried retained the ability to germinate to higher percentages than those dried over saturated salts, regardless of DAF (Fig 3.6). Developing embryos tolerated water contents as low as 0.10 g g^{-1} to varying degrees (60 to 100% germination) when flash dried. However, water contents below 0.10 g g^{-1} could not be achieved by flash drying; lower water contents were achieved by drying over salts. Extremely low germination (0 to 14%) was observed in developing embryos when dried over salts for 14 to water contents $\leq$ 0.10 g g^{-1} d (Fig 3.6). Conversely, germination increased for embryos held at 75% relative humidity for 14 d. Water content at this relative humidity averaged 0.22 $\pm$ 0.02 g g^{-1}.

At any harvest date between 240 and 397 DAF the ability of embryos to germinate in tissue culture after drying over saturated salts (RH 75 to 91%) decreased as

incubation time increased (Fig 3.7). However, less developed embryos (*i.e.*, 240 DAF) retained the ability to germinate to higher percentages after drying for longer periods than more developed embryos. Embryos held at high relative humidity (91%) for extended periods eventually turned brown and no germination was observed. Similarly, embryos held at conditions of low relative humidity ($\leq$ 32% RH) became grey after several weeks in tissue culture and did not enlarge or germinate.

Metabolic Rates in *P. remota* Slow Considerably During Development

Metabolism clearly slowed as the developmental program progressed in *P. remota* embryos (Fig 3.8). At 70 DAF the O_2 uptake was about 37 μmol/min/g DW and decreased to 0.05 μmol/min/g at shedding (397 DAF). Moreover, there is a strong correlation ($r = 0.97$) between the decrease in O_2 uptake and *in planta* water potential of developing embryos (Fig. 3.9).

The difference in O_2 uptake between freshly excised and PEG (-2.89 to -0.33 MPa) treated embryos was greater for more mature than less developed embryos. However, the metabolic rates of mature embryos never surpassed that of younger embryos at the same water potential during the experimental period (Fig 3.8). Similarly, increasing the water potential of mature *P. remota* embryos (397 DAF) to the same or higher levels than in mature pea axes (*c.* 90 DAF) did not result in greater O_2 consumption relative to pea axes (Fig 3.10). Metabolic rates in *P. remota* embryos are also remarkably low compared to recalcitrant and orthodox species at water contents between 0.20 and 2.10 g g^{-1} (Fig 3.11). Metabolic rates increased slightly when *P. remota* embryos were given a high temperature (35°C) pre-treatment.

TEAC Activity Peaks Before Mass Maturity Then Declines as Metabolic Rates Decline

Hydrophilic TEAC activity peaked before embryos reached maximum dry weight and then decreased progressively as metabolic activity declined. No lipophilic TEAC activity was detected throughout development (Fig. 3.12). There is sufficient evidence ($P < 0.0001$) to reject the hypothesis that hydrophilic TEAC activity remains constant as development proceeds. Duncan's Multiple Range test identified four homogeneous groups among hydrophilic TEAC activity throughout development, indicating that TEAC activity decreased significantly with the onset of maturation drying. Moreover, mature *P. remota* embryos (340 to 397 DAF) possessed more TEAC activity than either mature, hydrated pea or *A. hippocastanum* axes (Fig 3.12).

Discussion

Germination ability of excised *P. remota* embryos is gained early in development and precedes the ability to tolerate flash drying to relatively low water contents. Immature *P. remota* embryos are sensitive to desiccation, but increase in tolerance as they mature. At maturation drying (340 DAF) the critical water content is 0.16 g g^{-1}. This value corresponds to a critical water potential of -48 MPa. Nevertheless, embryos are sensitive to stresses imposed by the high levels of drying required for conservation in genebanks. Early in the developmental program of *P. remota* O_2 uptake rate are high, but at shedding metabolic activity is lower than that observed for some recalcitrant and orthodox tissues. Additionally, as respiratory activity decreases in *P. remota* embryos so does antioxidant activity. Therefore, metabolic activity is arrested during embryo development.

The acquisition of germination ability and desiccation tolerance in developing seeds has been the subject of many investigations. Several studies have shown that desiccation is a necessary developmental switch that triggers the onset of germination ability in orthodox seeds (Adams and Rinne 1981; Dasgupta and Bewley 1982; Kermode and Bewley 1985; Kermode 1995; Lehner *et al.* 2006). However, subsequent research on diverse species found that germination ability was acquired before desiccation tolerance (Bartels *et al.* 1988; Fischer *et al.* 1988; Fountain *et al.* 1989; Leprince *et al.* 1990; Sanhewe and Ellis 1996; Hay *et al.* 1997; Mai-Hong *et al.* 2003), that complete desiccation was not a prerequisite for germination ability (Kermode and Bewley 1989), and that germination ability is gained before abscission of vascular connections to the ovule (Galau *et al.* 1991). Studies performed on excised embryos or axes confirmed the hypothesis that germination ability is attained prior to desiccation tolerance for many species (Kermode and Bewley 1988; Leprince *et al.* 1993; Sun and Leopold 1993). This pattern was reported for excised embryos of *Elaeis guineensis* Jacq. (Aberlenc-Bertossi *et al.* 2003) and applies to embryos of *P. remota* as well. By 130 DAF *P. remota* embryos have the ability to germinate completely in tissue culture, but are damaged at water contents less than 1.69 g g^{-1}. It is not until mass maturity, approximately 230 -250 DAF, that embryos tolerate drying to 0.21 g g^{-1} or lower.

Although the onset of germination ability in *P. remota* coincides with decreases in water content, a sharp increase in fresh weight occurs *in planta* at 130 DAF (compare Fig. 3.2 to Fig 2.2). Total germination ability is gained 100 d before maximum dry weight is attained and approximately 200 d prior to maturation drying. Furthermore, tolerance to relatively low hydration (*e.g.* 0.20 to 0.16 g g^{-1}; -0.15 to -0.48 MPa) by rapid,

enforced drying is realized in conjunction with attainment of maximum dry weight but before maturation drying. The acquisition of desiccation tolerance, to low hydration levels, near or after maximum dry weight has been observed for such orthodox species as *Hordeum vulgare* L. (Bartels *et al.* 1988), *Digitalis purpurea* L. (Hay and Probert 1995), *Phaseolus vulgaris* L. (Sanhewe and Ellis 1996), and *Triticum aestivum* cv. Priokskaya (Golovina *et al.* 2001). Embryos of *E. guineensis* acquired desiccation tolerance about 30 d earlier than achievement of maximum dry weight (Aberlenc-Bertossi *et al.* 2003).

Seeds of recalcitrant (Hong and Ellis 1990; Tompsett and Pritchard 1993; Fu *et al.* 1994) and orthodox (Sun and Leopold 1993; Kermode and Finch-Savage 2002) species increase in desiccation tolerance, albeit to species-specific levels, throughout development. Likewise, *P. remota* embryos increase in desiccation tolerance as they mature. The main difference is that recalcitrant tissues lose viability at higher hydration levels than intermediate (Ellis *et al.* 1990, 1991; Pritchard 2004) or orthodox tissues (Vertucci and Leopold 1987; Farrant *et al.* 1988; Vertucci and Farrant 1995; Berjak and Pammenter 1997; Farrant *et al.* 1997; Walters *et al.* 2002). The critical water content for *P. remota* at the onset of maturation drying is 0.16 g g^{-1}. Similarly, germination of mature *P. dactylifera* seeds was reduced when dried to 0.14 g g^{-1} (von Fintel *et al.* 2004) and excised *E. guineensis* embryos were damaged at about 0.12 g g^{-1} (Grout *et al.* 1983; Ellis *et al.* 1991).

Critical water contents for recalcitrant *Theobroma cacao* L. embryonic tissues varied between 0.70 and 0.92 g g^{-1} (Liang and Sun 2000). Critical water contents for cotyledons and axes of *Inga* species were 0.67 and 1.22 g g^{-1}, these tissues were considered recalcitrant (Pritchard *et al.* 1995). Water contents less than 0.30 to 0.39 g g^{-1}

significantly reduced germination in seeds of *Bactris gasipaes* Kunth. (Arecaceae) (Bovi *et al.* 2004). No seeds of this presumably recalcitrant species (Flynn *et al.* 2004) survived water contents lower than 0.15 to 0.18 g g^{-1}.

When critical water content is plotted against development desiccation tolerance appears to be acquired continually and most rapidly during reserve accumulation (Walters 1998; Walters *et al.* 2002). However, because tissues at the same water content may have different water potentials, a more physiologically informative approach is the plot of critical water potential against development. In this way desiccation is acquired in discrete steps (Vertucci and Farrant 1995; Walters 1998; Sun and Liang 2001; Walters *et al.* 2002). Therefore, recalcitrant seeds are shed at relatively high water potentials and the corresponding critical water potentials reach a minimum around -20 to -15 MPa (Pritchard 1991; Farrant and Walters 1998; Walters 1998; Liang and Sun 2000; Sun and Liang 2001; Black and Pritchard 2002; Pritchard 2004; Walters 2004). At those water potentials tissues may succumb because they cannot recover from physical and/or physiological stresses imposed by drying (Leprince *et al.* 1993; Vertucci and Farrant 1995; Walters 1998; Walters *et al.* 2002). For example, upon drying to -15 MPa membrane bi-layers may de-mix and interact. Thus, when tissues are re-hydrated organellar bounding layers may not assume their original configuration and lethal damage may occur (Wolfe 1987; Steponkus *et al.* 1993; Webb *et al.* 1994). Furthermore, recalcitrant tissues may survive to a hydration level where unregulated metabolism can occur and their demise is due to accumulation of deleterious metabolic by-products (Farrant *et al.* 1988; Leprince *et al.* 1993; Berjak and Pammenter 1997; Farrant and Walters 1998; Walters *et al.* 2002).

Alternatively, tissues with intermediate and orthodox physiologies can tolerate and recover from stresses imposed at lower water potentials (Vertucci and Roos 1990, 1993; Vertucci and Farrant 1995; Walters 1998; Sun and Liang 2001). Coffee species, for example, generally demonstrate critical water potentials intermediate to recalcitrant and orthodox seeds (Dussert *et al.* 1999; Sun and Liang 2001).

Even before shedding, *P. remota* embryos reach a critical water potential that is intermediate to that observed for recalcitrant species such as *A. hippocastanum*. (Farrant and Walters 1998) and orthodox species like pea (Vertucci and Roos 1990). Although the critical water potential for *P. remota* embryos could not be calculated at shedding presently, previous work has demonstrated that the *in planta* water potential of embryos is about -60 MPa (see Chapter 2). Presumably, the critical water potential of embryos at shedding would be at least -60 MPa or lower.

Embryos of *P. remota* can tolerate and recover from the types of desiccation stresses imposed when dried into hydration level 2, which corresponds to water potentials between about -150 to -11 MPa (Vertucci and Farrant 1995; Walters *et al.* 2002; Pritchard 2004). At this hydration level most of the hydration or 'bulk' water has been removed, recalcitrant tissues cannot tolerate removal of this type of water (Pammenter *et al.* 1991; Finch-Savage 1992; Vertucci and Farrant 1995). Protection from the stresses encountered at this hydration level in *P. remota* may be due in part to the high levels of sucrose, some amounts of trehalose, and other oligosaccharides accumulated during development (see Chapter 2). Sucrose and supplementing oligosaccharides are thought to stabilize membranes and macromolecules during drying; thereby protecting these systems from desiccation damage (Leprince *et al.* 1993; Steadman *et al.* 1996; Obendorf 1997;

Corbineau *et al.* 2000; Greggains *et al.* 2000; Bailly *et al.* 2001; Bryant *et al.* 2001; Kermode and Finch-Savage 2002; Walters *et al.* 2005). Additionally, *P. remota* embryos appear to have systems in place to control metabolism and offer protection against free-radical attack (see below).

The higher germination observed for *P. remota* embryos dried over N_2 compared to those dried over saturated salts may be due to differences in drying rates between the treatments. The minimum water content of embryos dried over N_2 is achieved by 24 h; whereas embryos dried over salts did not reach equilibrium for 3 to 7 d. Seeds or embryonic tissues dried rapidly most likely avoid viability loss because they are not maintained at hydration levels where deleterious metabolic reactions (Pammenter *et al.* 1998; Leprince *et al.* 2000; Walters *et al.* 2001) or mechanical stresses (Leprince *et al.* 2000; Liang and Sun 2000) take place.

As a first approximation, the decreased capacity for maturing embryos to germinate after exposure to various drying stresses seems to conform to the hypothesis that maximum longevity is achieved near the point of maximum dry weight (*i.e.* 240 DAF) and then declines (Harrington 1972; TeKrony and Egli 1995). This is contradictory to results obtained for barley (Ellis and Pieto Filho 1992); tomato (Demir and Ellis 1992; Ellis *et al.* 1992); and foxglove (Hay and Probert 1995) that show seed quality improved during desiccation.

However, the possibility that the results from this study are confounded by dormancy within *P. remota* embryos (see Chapter 4) cannot be ignored. Sundstrom (1990) reports that low seed moisture treatments induced greater dormancy in *Capsicum annum* L. seeds. It may very well be that the most mature embryos do not germinate

because they were not exposed to dormancy breaking conditions. As a consequence embryos fail to grow and ultimately germinate. At the same time viability is lost due to harmful effects of seed aging (Priestley 1986).

Likewise, the progressive loss of viability in embryos held at higher relative humidity can be ascribed to aging effects (Priestley 1986; Walters 1998; Walters *et al.* 2001). *P. remota* seeds/embryos must be well into hydration level five, a level where water potentials exceed -1.7 MPa (Vertucci and Farrant 1995), before continued growth and germination occurs (see Chapter 4). Therefore, when embryos are held at 91% or 85% RH they are at hydration levels that prevent further growth but allow for deterioration over time. At much lower relative humidity, however, the desiccation stresses are detrimental and are not resolved upon rehydration. However, drying over 75% RH seems to be the level to which embryos can be stressed and maintain some degree of viability, although longevity under these conditions is comparatively short (Sacandé *et al.* 2000; 2000).

It is evident that metabolism in *P. remota* embryos 'shuts-down' during development (Figs. 3.8 and 3.9). This is consistent with observations for orthodox seeds (Farrant *et al.* 1997). Controlled down-regulation is exerted through maturation and drying *in planta* and may be the result of the high sucrose concentrations found throughout development (Leprince *et al.* 1992). Interestingly, metabolic rates slow considerably before embryos obtain their maximum dry weight or gain tolerance to maximum desiccation. Also, it is remarkable that respiratory activity is so comparatively low (see Figs. 3.9 and 3.10) even if water potential of embryos is elevated to hydration levels 4 and 5 (Figs. 3.8 and 3.10).

For instance, the current hypothesis is that respiratory activity continues until tissues are dried below 0.25 g g^{-1} or -11 MPa, implying that respiratory pathways are active to various degrees above these hydration values. This certainly has been the case for many recalcitrant and orthodox species (Vertucci and Leopold 1984, 1987, 1987; Vertucci 1989; Leprince *et al.* 1999; Walters *et al.* 2001). However, even at relatively high water contents (*i.e.* 1.6 g g^{-1}) metabolic rates in *P. remota* embryos give the impression of inactivity. When embryos are exposed to dormancy breaking temperatures (35°C) then metabolic activity rises (Fig 3.11). Suggesting that mature *P. remota* embryos require very high water potentials and/or some other factor such as increased temperature (see Chapter 4) to promote metabolic activity. The ability to highly regulate metabolism suggests that, in spite of dormancy, further reductions in hydration may be possible. Moreover, the ability to regulate metabolic rates is beneficial from an ecological context (Garwood and Lighton 1990), as *P. remota* dispersal units can remain dormant for some time (see Chapter 4).

Buitink *et al.* (2002) questioned whether free radical processing systems function at reduced hydration conditions *in vivo*. The evidence presented here indicates that indeed antioxidant systems are active during maturation and function at the relatively low hydration levels (*c.* 0.40 g g^{-1}; -60 MPa) found in mature *P. remota* embryos. Similarly, antioxidant enzymes were active, in varying proportions during seed development in *Phaseolus vulgaris* cv. Vernel (Bailly *et al.* 2001) and *Vicia faba* (Arrigoni *et al.* 1992).

Several other features of antioxidant systems in *P. remota* embryos stand out. For example, hydrophilic antioxidant activity peaks before the onset of mass maturity and then again before drying *in planta*. During these developmental stages embryonic water

potential ranges between about -0.7 and -3.0 MPa. Presumably, these increases in antioxidant activity occur to protect embryos from potential upcoming free radical attack that are mediated by lower water potentials (Vertucci and Farrant 1995).

Next, the antioxidant activity of *P. remota* embryos is very high compared to axes of pea and *A. hippocastanum*. This is somewhat surprising, as one would expect the more metabolically active tissues to also have higher antioxidant activities. However, the developmental program in *P. remota* is considerably longer than in either pea or *A. hippocastanum*. It is reasonable then to expect *P. remota* to have higher antioxidant capacity as the embryos may be at water potentials where metabolism is consistently active for longer periods of time than in other species.

Also, no lipid soluble antioxidants could be detected with the present assay. However, some balance of hydro- and lipophilic antioxidants are thought to confer maximum desiccation tolerance (Leprince *et al.* 1993; Greggains *et al.* 2000; Bailly 2004). Nevertheless, if there are little to no lipophilic antioxidants present then protection from membrane-attacking reactive oxygen species cannot be guaranteed. This may be one reason why *P. remota* embryos do not seem to display extreme levels of desiccation tolerance.

To summarize, *P. remota* embryos are not considered recalcitrant because the critical water content drops below -15 MPa and metabolic activity shuts down as development progresses. Toxic by-products that may be produced, despite remarkably low levels of respiration, are controlled by high antioxidant activity throughout development. It seems, however, that embryos are not tolerant of extreme drying. Therefore, until the effect of enforced desiccation on embryo dormancy mechanisms can

be elucidated further the feasibility of storage using conventional genebank conditions remains questionable. In the meantime, efforts to store *P. remota* in *ex situ* collections should focus on methods used to conserve tissues with intermediate and recalcitrant physiologies, such as cryo-preservation. This method has been successful for preservation of other palm species (Grout *et al.* 1983; Al-Madeni and Tisserat 1986; Engelmann *et al.* 1995).

Figures

Fig 3.1. Germination of young (130 DAF) *Pritchardia remota* embryo in tissue culture.

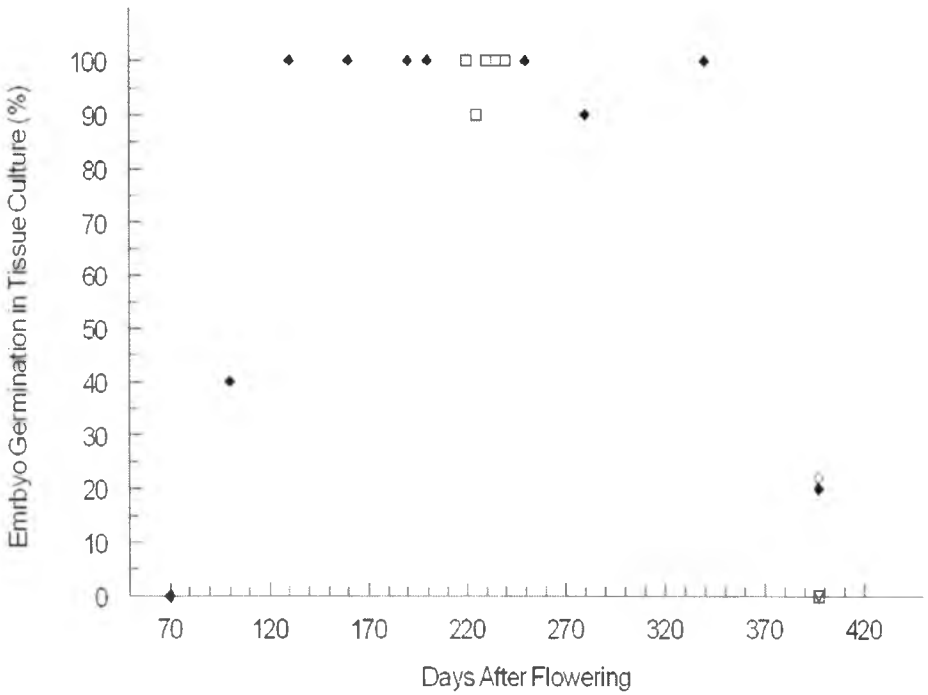

Fig. 3.2. Germination of developing *Pritchardia remota* embryos in tissue culture from seeds collected from a single tree at the University of Hawaii at Manoa in the 2004-05 season (closed diamonds), a single harvest from the previous tree in 2005 (open diamond), multiple trees growing in Waimanalo, Oahu during 2005 (open squares), and a single harvest from two different trees than the previous growing on the university campus in 2005 (open, inverted triangle).

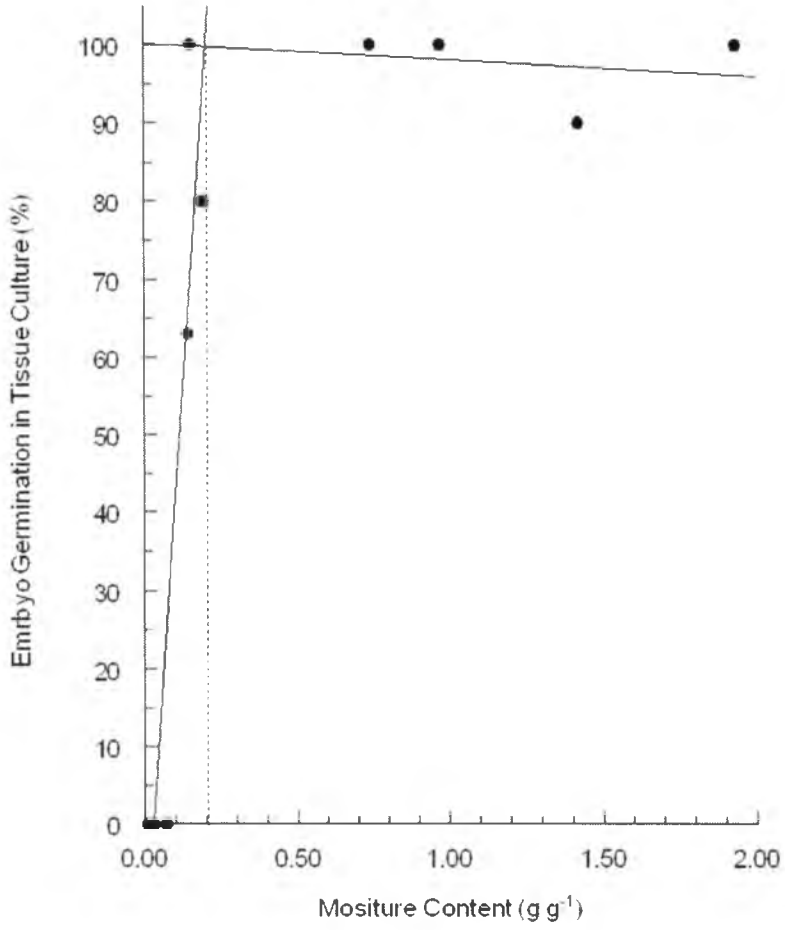

Fig. 3.3. A typical graph of decreasing germination with drying used to calculate the critical water content of developing *P. remota* embryos. A vertical line is drawn from the break point in the germination-water content curve to the x-axis. This point is taken as the critical water content.

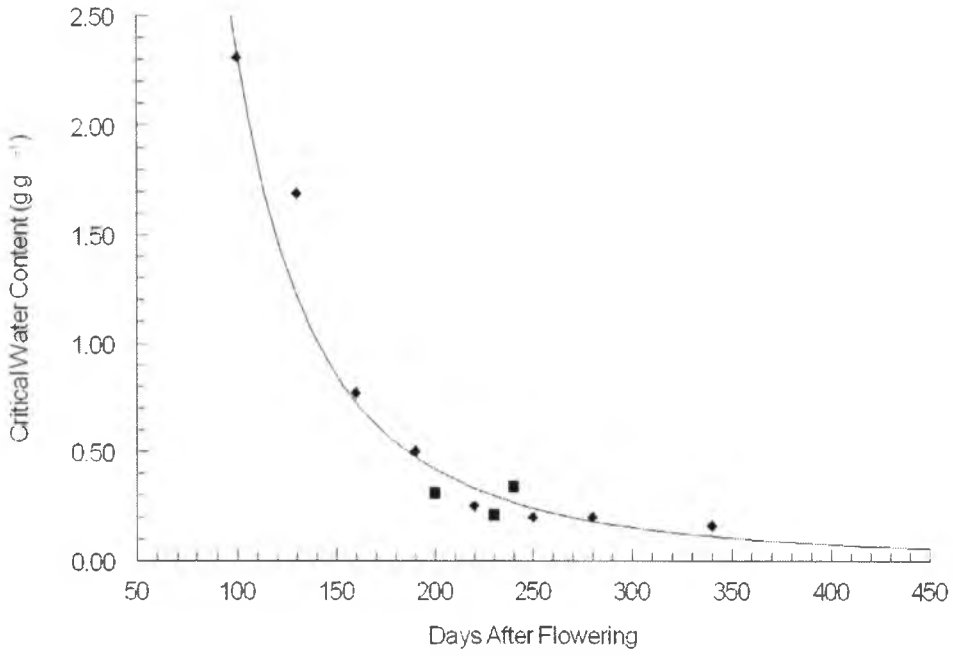

Fig 3.4. The critical water contents for developing *Pritchardia remota* embryos collected in 2004-05 from a single tree at the University of Hawaii at Manoa (diamonds) and several trees from Waimanalo, Oahu in 2005 (squares). Critical water contents decreased from 2.31 g g^{-1} at 100 DAF to 0.16 g g^{-1} at 340 DAF. The regression equation is $Y = 212940(X)^{-2.48}$, $R^2 = 0.92$.

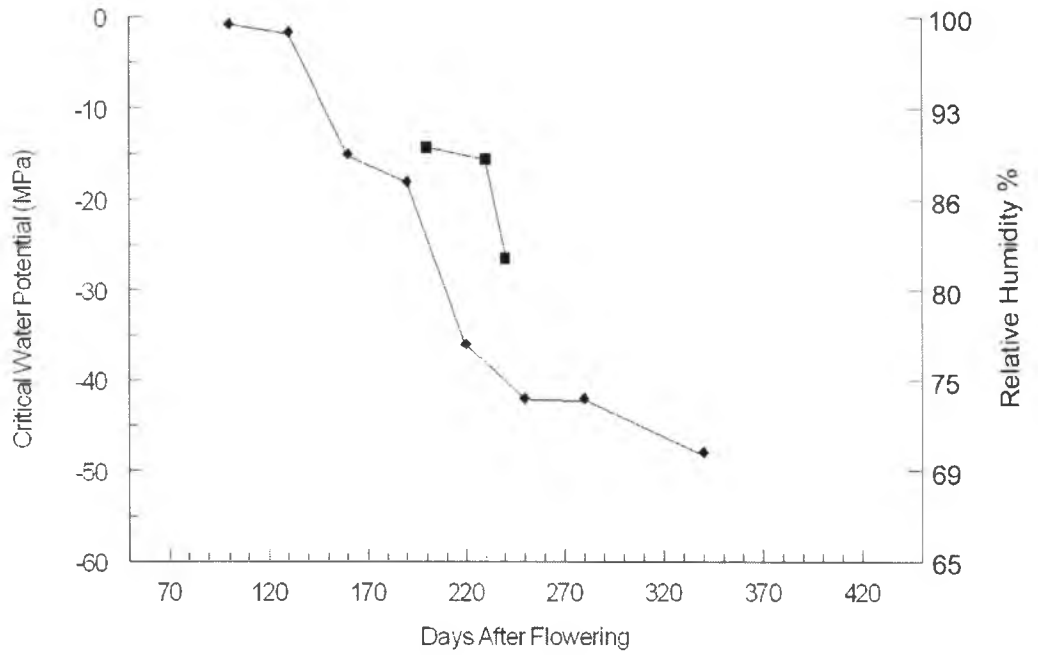

Fig 3.5. Critical water potentials calculated for developing *Pritchardia remota* embryos. Symbols as in Figure 4.

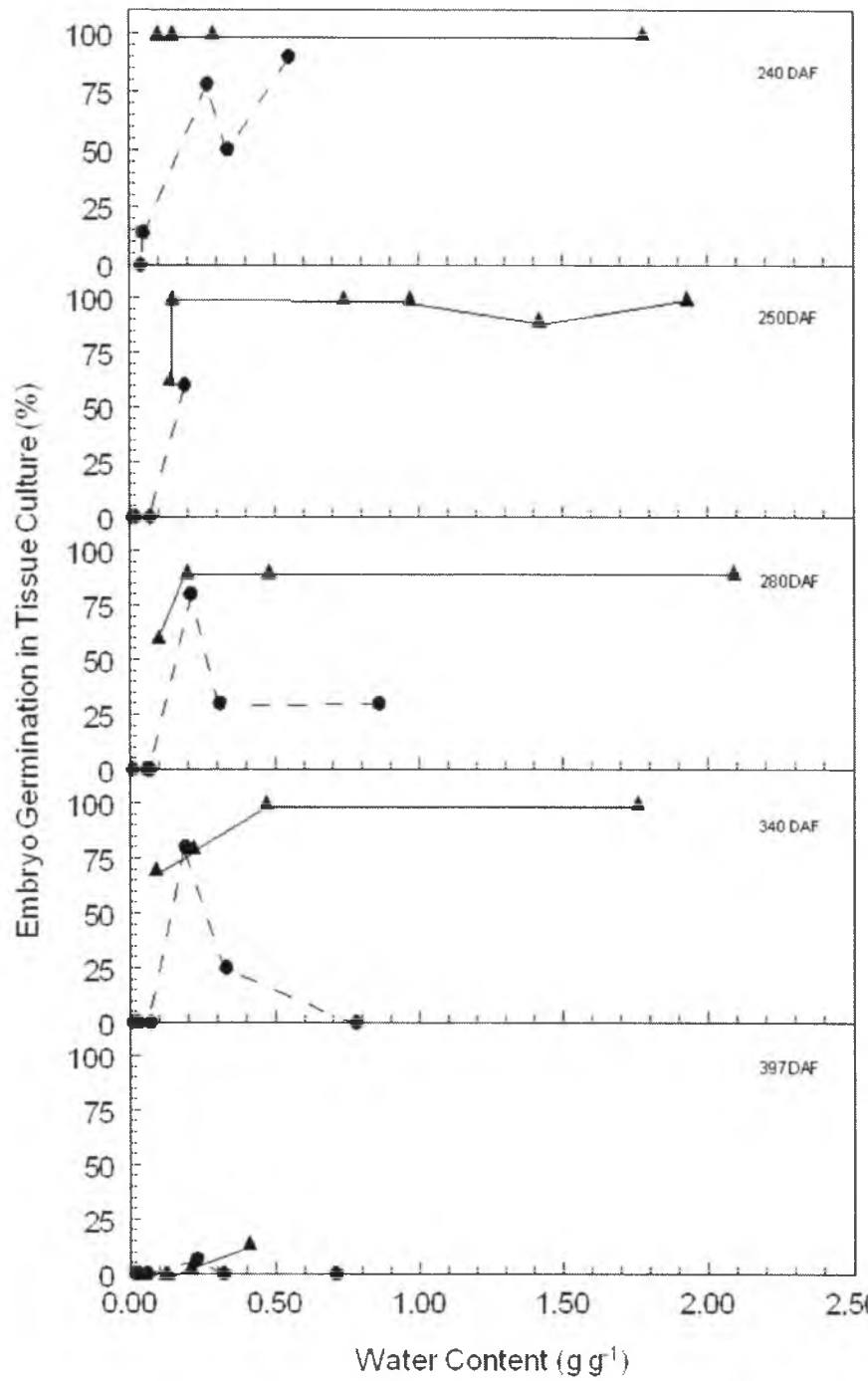

Fig 3.6. Germination of developing (240 to 397 DAF) embryos of *Pritchardia remota* 'flash' dried over a stream of nitrogen gas (triangles) for 0 to 1200 min. or over various saturated salt solutions at 25°C for 14 d (circles).

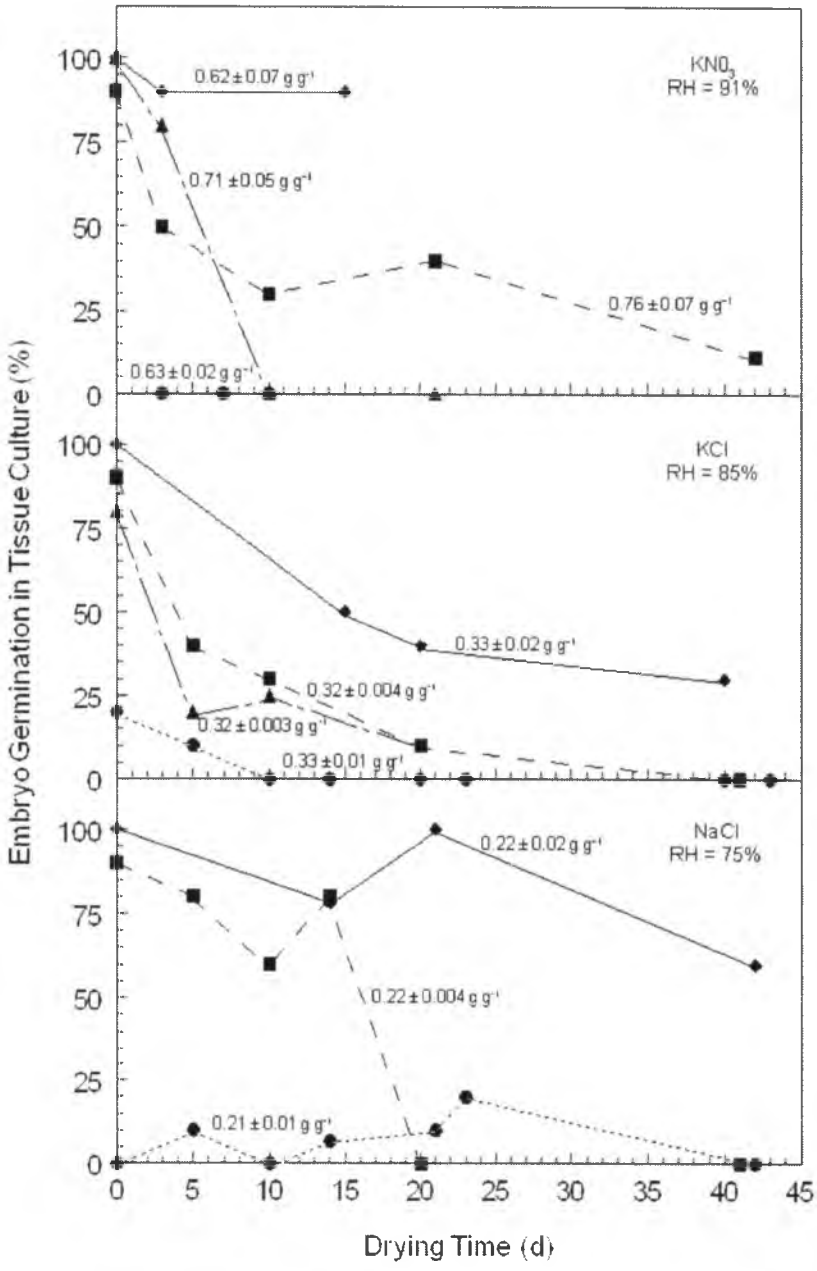

Fig. 3.7. Germination of developing *Pritchardia remota* embryos after drying over saturated solutions of RH = 91, 85 or 75%. Embryos were isolated from seeds harvested at 240 (diamonds), 280 (squares), 340 (triangles), and 397 (circles) DAF.

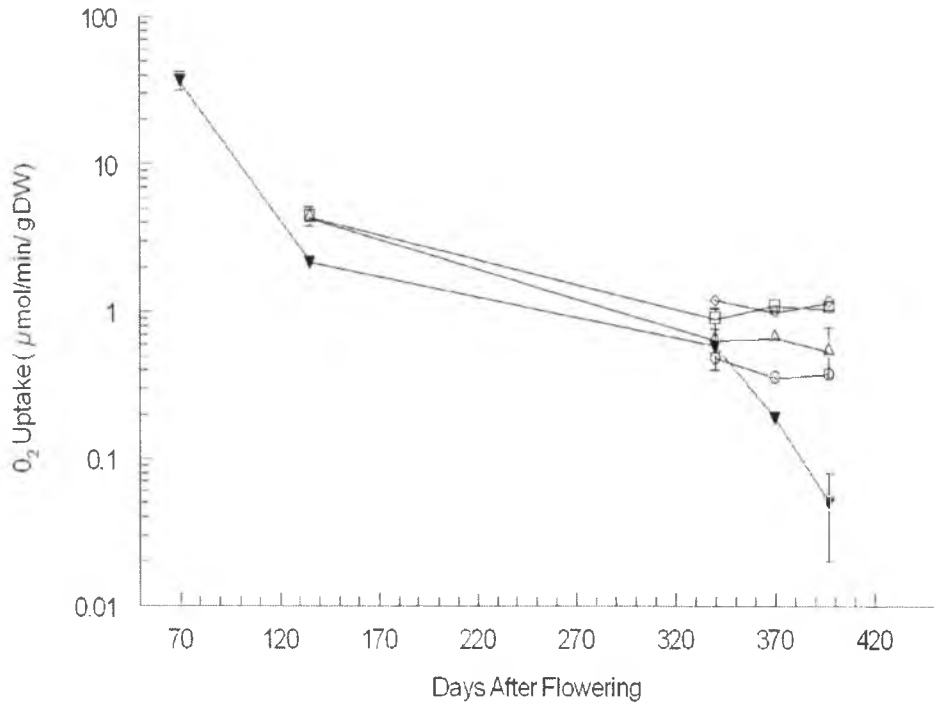

Fig 3.8. Comparison of *in planta* (solid, inverted triangles) respiration of developing embryos to respiration of developing embryos imbibed in PEG solutions (open symbols) of -0.33 (diamonds), -0.77 (squares), -1.45 (triangles), and -2.89 (circles) MPa for 24 h at 25°C. Error bars denote SE.

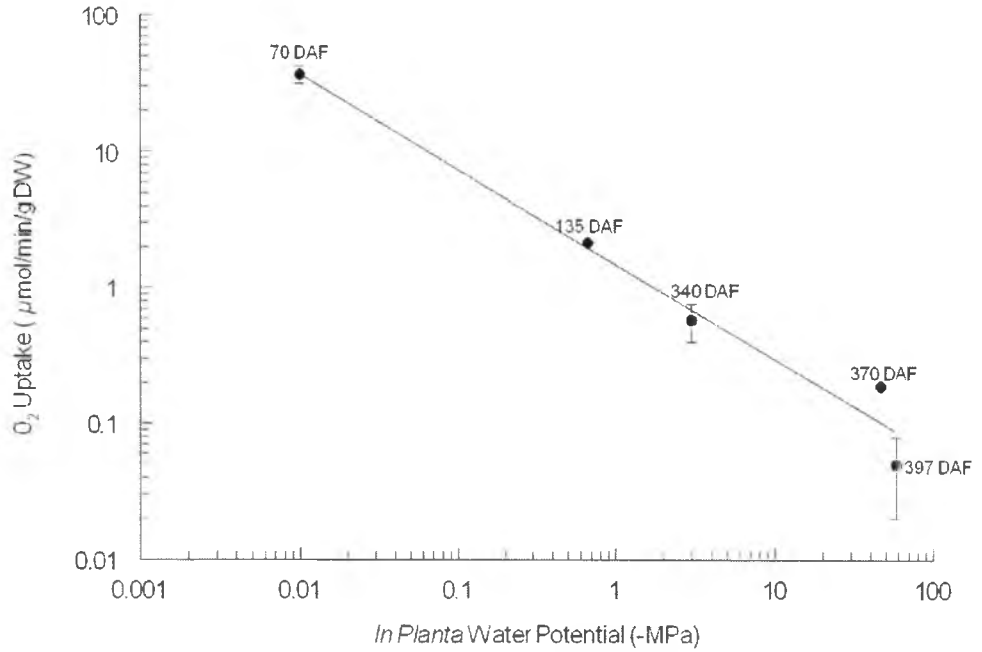

Fig 3.9. Relationship between *in planta* water potential of developing *Pritchardia remota* embryos and oxygen uptake. Error bars denote SE. $Y = -1.42 - 8.05\ln(X) + 4.59\sqrt{X}$, r = 0.97.

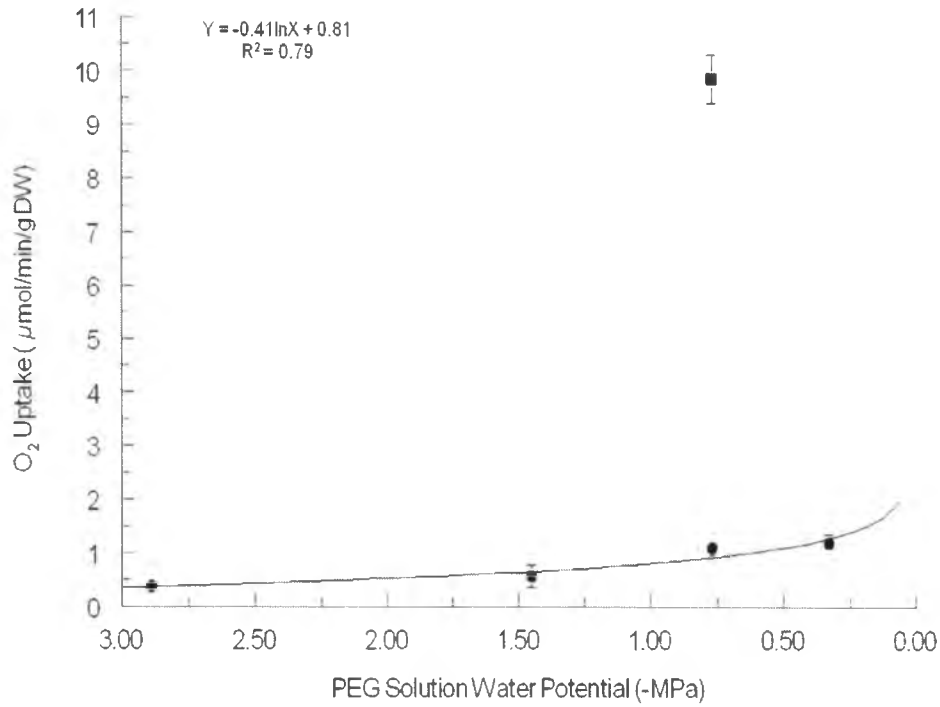

Fig. 3.10. Comparison of respiration between 397 DAF *Pritchardia remota* embryos imbibed in various PEG solutions (circles) and mature pea axes imbibed in PEG solution with a water potential of -0.77 MPa (square) for 24 h at 25°C. Error bars denote SE.

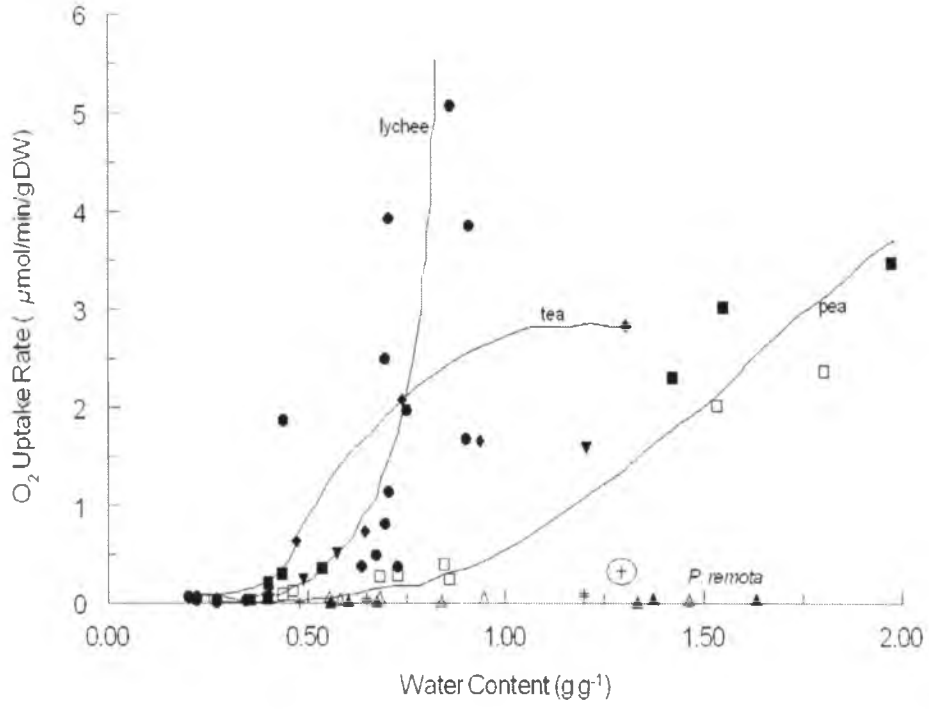

Fig 3.11. A comparison of respiration between recalcitrant Lychee (circles) and Tea (diamonds) axes, orthodox pea (squares) axes, and mature *Pritchardia remota* embryos (triangles). Closed and open triangles represent PEG and flash dried embryos, respectively. The circled point represents *P. remota* embryos exposed to 35°C before measurements were taken at 25°C. Open squares are measurements taken concurrently with *P. remota.*

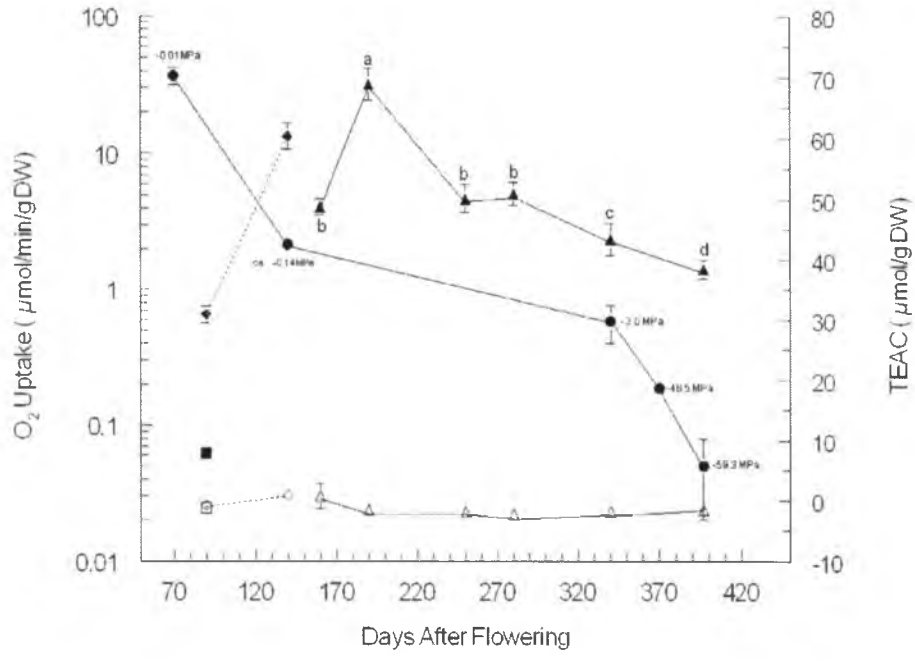

Fig. 3.12. Trolox equivalent antioxidant capacity of developing *Pritchardia remota* embryos (triangles), mature pea axes (squares), and *Aesculus hippocastanum* axes (diamonds). Open and closed symbols are hydrophilic and lipophilic fractions, respectively. Circles signify respiration in *P. remota* embryos. Error bars indicate SE. Letters denote significant differences at $\alpha = 0.05$ using Duncan's Multiple Range test.

CHAPTER 4

DORMANCY MECHANISMS IN SEEDS OF *PRITCHARDIA REMOTA* (KUNTZE) BECC., AN ENDANGERED PALM ENDEMIC TO HAWAII

Introduction

The objectives of this study were to determine if (1) germination is delayed in drupes of *Pritchardia remota* (Kuntze) Becc.; (2) the endocarp and seed coat are permeable to water; (3) covering structures delay embryo emergence and therefore radicle protrusion; and (4) embryos are underdeveloped at shedding. Emergence of *Pritchardia* spp. seedlings is known to be slow and anecdotal evidence suggests that the seeds are dormant. However, a clear understanding of the possible seed dormancy mechanism(s) in this species is lacking. *Pritchardia remota* is a federally listed endangered species. Consequently, understanding mechanisms that control germination may assist in meeting the recovery objectives for this species (U.S.F.W.S. 1998).

How Long Does It Take for Palm Seeds to Germinate?

Although seeds of a few palm species germinate in 7 to 28 d (Loomis 1958; Brown 1976; Wagner 1982; von Fintel *et al.* 2004), the majority of species germinate slowly (Loomis 1958; Jordan 1970; Basu and Mukherjee 1972; Wagner 1982; de Carvalho *et al.* 1988; Broschat and Meerow 2000). Koebernik (1971) lists 126 species that complete germination more than 100 d after sowing. Likewise, Tomlinson 1990 (in Broschat and Meerow 2000) estimates that 25% or more of all palm species require more than 100 d to germinate.

Estimates of germination rates vary widely for *Pritchardia* spp. For instance, emergence of shoots from *P. lowreyana* fruits occurred 45 d after sowing (Loomis 1958).

Germination for three *Pritchardia* species was complete between 40 to 77 d after sowing (Koebernik 1971). Germination for seven species of *Pritchardia* commenced 28 to 308 d after planting (Manokaran 1979; Wagner 1982). Time for 50% of seeds to germinate for *P. hillebrandii*, *P. thurstonii*, and *P. pacifica* equaled 1.9, 2.1, and 9 weeks, respectively (Yoshii and Rauch 1989; Rauch 1994; Maciel and Mogollón 1995). The range in germination data may be due to different definitions of what constitutes germination among these authors.

Environmental Factors That Influence Dormancy and Germination in Palms

Temperature is perhaps the major factor governing the alleviation of dormancy and promotion of germination in palms. Early work on *Elaeis guinnensis* Jacq. indicated that a high-temperature pre-treatment (39.5°C) would improve germination when seeds were later transferred to lower temperatures (Hussey 1958; Rees 1961, 1962). Work by Carpenter (1987; 1988; 1988; 1989) and Carpenter *et al.* (1988; 1989; 1993) suggests that the optimal temperature range for germination of many palms falls narrowly around 35°C. Generally, germination occurs more slowly and to lower percentages at temperatures $\geq$ 40°C or $\leq$ 25°C (Brown 1976; Robertson and Small 1977; Broschat and Donselman 1986; Broschat and Meerow 2000).

Typically, no germination is observed at relatively low temperatures (*i.e.* $\leq$ 15°C) (Broschat and Meerow 2000). However, a positive cold stratification response has been shown for a few palms thus far (Carpenter 1988; Orozco-Segovia *et al.* 2003). In some palms alternating temperatures improved germination rate and percent. However, germination of *Sabal palmetto* (Jacq.) Pers. was less uniform and lower in response to

alternating temperatures, which simulated environmental conditions, than constant temperatures (Brown 1976).

Seeds of various palms respond differently to light and darkness. For example, light inhibited germination of *S. palmetto*. Germination in the dark was five times greater and began 15 d earlier than in light (Brown 1976). *Aiphanes aculeata* Willd. seed germination did not differ in light or darkness (Silva *et al.* 1999); however, light was a requirement for germination in seeds of *Hyophorbe lagenicaulis* (L. Bailey) H.E. Moore (Wood and Pritchard 2003).

Dormancy Mechanisms in Palm Fruits and Seeds

Seeds of fruits with water-impermeable coats are said to be physically dormant. In most cases, impermeability of the fruit or seed coat is due to a layer(s) of lignified and/or sclereified cells (Li *et al.* 1999, 1999; Baskin and Baskin 2001) or multiple palisade layers (Meisert 2002). It is often assumed that the thick endocarps and seed coats of many palms are impermeable to water. For example, *Hyphaene thebaica* (L.) Mart. possesses a thick pericarp that is presumably impervious (Moussa *et al.* 1998). Yet, these authors provide no evidence that imbibition is blocked due to the pericarp. On the contrary, imbibition may occur albeit relatively slowly (Robertson and Small 1977; Ehara *et al.* 2001; Wood and Pritchard 2003) or rapidly (Brown 1976), suggesting that the fruit or seed coats are permeable. Developing endocarps and seed coats of *Pritchardia pacifica* showed no signs of lignification, although the inner layer of the endocarp possessed 2-4 layers of thick-walled cells (Reddy and Kulkarni 1982).

Studies on the effects of mechanical scarification on palm seeds indicate that this treatment significantly increases germination (de Dios Holmquist and Popenoe 1967;

Doughty *et al.* 1986; Moussa *et al.* 1998). For example, seeds, with the exocarp removed but presumably the meso- and/or endocarp intact, of *Archontophoenix alexandrae* (F. Muell.) H. Wendl. & Drude and *Ptychosperma macarthurii* (H. Wendl. ex Veitch; H. Wendl. ex Hook. f.), were mechanically scarified with a file until the endosperm was visible. This treatment resulted in improved germination compared to controls (Nagao *et al.* 1980). However, studies regarding mechanical scarification of palm seeds have not investigated whether imbibition of scarified seeds is improved compared to non-scarified seeds. Therefore, it is not clear if mechanical scarification actually decreases mechanical resistance of covering structures or improves imbibition.

Physical restriction of embryo growth is considered to be one cause of non-deep physiological dormancy (Baskin and Baskin 2001). Physical restriction may be caused by embryo covering structures such as opercula, endosperm, and seed coats. Furthermore, fruit coats may mechanically restrict growth of the embryo (Bewley and Black 1994; Baskin and Baskin 2001). Seeds of various palms are known to possess opercula and the majority of fruits are single seeded drupes with stony endocarps (Uhl and Dransfield 1987). Opercula provide mechanical resistance to embryo elongation in *E. guineensis* (Hussey 1958). In de-operculated seeds, embryos commenced elongation after 4 days at 30°C. Embryo elongation in de-operculated seeds was enhanced if seeds received a high temperature pre-treatement or were exposed to pure oxygen. However, intact seeds failed to germinate for six months. Similarly, embryo elongation in *Rhapidophyllum hystrix* (Pursh) H. Wendl. & Drude was controlled by the operculum (Carpenter and Cornell 1993). Moreover, removal of the pericarp, especially the

endocarp, significantly improved germination in a variety of palms (Broschat and Donselman 1986; Briceño and Maciel 2004).

Application of gibberellins alleviates non-deep and intermediate levels of physiological dormancy in many seeds, perhaps by substituting for warm or cold stratification (Baskin and Baskin 2001). Gibberellins may also stimulate the production of enzymes that degrade cell walls, thus reducing mechanical resistance to radicle protrusion (Sánchez *et al.* 1990; Bewley and Black 1994). Germination became more rapid and uniform when seeds of *A. alexandrae* and *P. macarthurii* were immersed in 100 or 1000 ppm GA for 24 or 72 h (Nagao and Sakai 1979; Nagao *et al.* 1980). However, neither final germination percent nor time to 50% germination was different compared to controls in seeds of *Chrysalidocarpus lutescens* H. Wendl. or *Chamaedorea seifrizii* Burret exposed to GA (Yoshii and Rauch 1989).

Hussey (1958) postulated that embryos of *E. guineensis* needed to produce pectic enzymes, to degrade the middle lamella of endosperm cells that formed part of the operculum, for a reduction in mechanical restraint. A correlation between pectin methylesterase activity and dormancy (caused by mechanical restriction by the megagametophyte) release was found in *Chamaecyparis nootkatensis* (D. Don) Spach. Gibberellic acid promoted activity of this enzyme during the germinative process (Ren and Kermode 2000). Moreover, endo-βmanannase and β-mannosidase are found in the endosperm of *Phoenix dactylifera* L., with activity of β-mannosidase detectable before germination (DeMason 1985).

A distinguishing feature of most palms is their small, late-maturing embryo (Uhl and Dransfield 1987). These underdeveloped embryos require further growth, outside of

the seed and endocarp, before germination is complete; hence they are considered morphologically dormant (Baskin and Baskin 2001). Analysis of Wood and Pritchard's (2003) data shows that embryos of *H. lagenicaulis* are 10.3% the length of seeds at shedding. Furthermore, the cotyledonary stalk (referred to as a coleorhiza-like organ), which contains the apical and root meristems, must continue growing past the seed coat before radicle protrusion occurs. Continued embryo growth before radicle protrusion has been documented for palms such as *E. guinnensis* (Hussey 1958), *P. kaalae* (Hodel 1977), *Eugeissona tristis* Griff. (Fong 1978) and *Metroxylon sagu* Rottb. (Ehara *et al.* 1998).

Germination studies of excised *P. remota* embryos in tissue culture suggest that embryos become dormant after the onset of maturation drying (see Chapter 2). Therefore, to determine if germination of *P. remota* is delayed due to dormancy mechanisms, fully mature fruits were harvested and submitted to germination studies at various temperatures. Moreover, germination studies were conducted on seeds with the pericarp removed at the same conditions. Endocarp and seed coat anatomy was also investigated. Furthermore, embryo length, moisture content, and water potential were measured throughout the germination process. Finally, germination tests were performed on seeds with the operculum removed or left intact and after immersion in GA_3.

Materials and Methods

Plant Material

Pritchardia remota naturally occurs in two populations on Nihoa (23°3'N, 161°55'W), a small, uninhabited island in the northwest Hawaiian island chain, approximately 450 km from Honolulu, HI (21°18'N, 157°49'W). These populations

comprise the *Pritchardia* coastal forest on Nihoa (Wagner *et al.* 1999). *Pritchardia remota* is a single-trunked palm about 4-5 m tall with unarmed petioles, costa-palmate leaves, and paniculate inflorescences. Fruits are single-seeded drupes (Fig. 4.1) and the endospermic seeds possess a relatively thick coat. The linear embryo comprises less than 1% of total seed volume (Pérez unpublished data). The fresh mass of seeds and embryos at shedding is 2.1 ± 0.1 g (mean ± SE) and 7.7 ± 0.42 mg (mean ± SE), respectively (see Chapter 2). Endocarps remain intact upon shedding, but the epicarp may be intact or broken. Gravity is the primary dispersal mechanism.

Fruits at the shedding stage (*c.* 397 DAF) were harvested, from three trees growing on the grounds of the University of Hawaii at Manoa (Honolulu, Oahu), at weekly intervals by gently shaking infructescences or collecting fruits that had been shed naturally. All harvests took place from September to October 2005 and fruits from the different trees were combined. If necessary, fruits were stored in a single layer in open trays or polypropylene bags at room temperature (*c.* 20 – 23°C) for no more than one week.

Germination Studies

'Move-along' Experiments Adapted to Coastal Hawaiian Climate

Five replicates of 10 intact fruits were sown in a 5:1 coral sand to distilled water (v:v) mixture within clear plastic trays (15 x 12.5 x 4.5 cm), with tight fitting lids. Fruits were placed lengthwise in the sand and buried to their mid-line. To allow gas exchange, the lids were punctured once with a dissecting needle. We placed trays in incubators (I-30VL, Percival Scientific, Perry, IA) set at constant 35°, 25°, or 15°C or alternating 28.5/22°C (dry-season) or 26/19.5°C (wet-season). High and low temperatures were set

to alternate every 12 h. The alternating temperature regimes were used to simulate conditions during the dry and wet-season on Nihoa, in order to determine if dispersal units require warm stratification.

Each chamber was set to a 14 h alternating photoperiod (80 μmol m^{-2} s^{-1}, cool white fluorescent light, F17T8 TL741, Philips Electronics, Eindhoven, Netherlands). Lights came on 1 h before the warm cycle and stayed on for 1 h after the cool cycle in treatments with alternating temperatures. A randomized complete block design was used for this and all further experiments in the incubators. Treatments were blocked on potential light and temperature gradients within the chambers.

After 13 weeks, trays held at 28.5/22°C were shifted to 26/19.5°C and those at 26/19.5°C were moved to 28.5/22°C. Control trays for these treatments remained at the original temperatures. Germination counts were conducted every week. For this experiment, germination was scored as coleoptile or radicle emergence through the mesocarp. No attempts to control fungal contamination were made in this experiment.

This experiment was terminated after 26 weeks. At that time, seeds were extracted from any remaining fruits, checked for embryos with obvious signs of fungal contamination and discarded. Embryos not appearing gray and flaccid were soaked in 2,3,5-Triphenyl-2H- Tetrazolium Chloride (TTC) for 24h at 30°C then checked for completeness of staining under a dissecting microscope.

Imbibition Studies

Imbibition of Excised Embryos, Seeds, and Endocarps

Imbibition studies were conducted on excised embryos, intact seeds with the pericarp removed, and endocarps with the epi- and mesocarp removed, but seeds

remaining inside. Imbibition studies were carried out on excised embryos because they make up a small proportion of the whole seed. Therefore, even small increases (*e.g.* 0.5 – 1.5%) in seed fresh mass during imbibition studies, which could be interpreted as surface wetting instead of imbibition, can obscure large increases in embryo fresh mass.

Embryos were excised according to Pérez (2005). Embryos were placed in 10 cm Petri dishes on blotter paper (Steel Blue, Anchor Paper, St. Paul, MN), moistened with 5 ml of distilled water. After 1, 2, 3, 4, 5, 6, 7 h and every 24 h for 168 h embryos were removed from the dishes, gently rolled in dry paper towels and weighed. From seven hours onward, the Petri dishes were sealed with two layers of plastic film.

Seed were removed from fruits by cracking the endocarp with pliers. Seeds with obvious signs of damage were not used. The epi- and mesocarp of fruits was removed with a knife using a scraping motion. Fruits were not soaked in water prior to removal of the epi- and mesocarp. The fresh mass of seeds and endocarps was measured as described for embryos. However, measurements for these treatments extended to 336 h. Additionally, the fresh mass of embryos excised from seeds and endocarps after 336 h of imbibition was measured.

Effect of Endocarp on Imbibition

In this experiment two levels of endocarp (presence or absence) and imbibition times (0 or 4 weeks) were arranged as a factorial. The fresh mass of embryos, seeds with embryos removed, and, if applicable, cleaned endocarps without seeds were measured. All samples were dried at 95°C for 96 h then dry mass was determined gravimetrically. Water content was calculated from fresh and dry mass and is stated as g H_20 per g dry mass (g g^{-1}).

Mechanical Scarification Effects on Imbibition of Seeds and Endocarps

Seeds were mechanically scarified with a cordless rotary tool (Mini-Mite 750-02, Dremel Inc., Racine, WI) set to high speed and coarse sanding drum attachment. Seed coats were mechanically scarified for five seconds at a point away from the operculum, scarified for three seconds at the junction between the operculum and seed coat, or not scarified. The endosperm was visible in all scarified seeds. In a separate trial, endocarps remained intact or were scarified for five seconds. The increase in fresh mass of seeds and endocarps at 20 min, 1, 2, 3, 4, 5, 6, and 12 h, and every 24 h for 336 h was measured.

For each type of imbibition study five seeds or endocarps were replicated four times and blocked on potential temperature differences along the lab bench. The average temperature in the lab was 23.2°C ± 0.1 (mean ± SE). Within each experiment seeds or endocarps were placed in trays (described previously) containing 35 ml of distilled water. This amount of water only covered about ¼ of the surface of seeds or endocarps. Portions of seeds or endocarps that had been mechanically scarified were always submerged. Water within the trays was changed every 48 h. The increases in fresh mass were converted to percentages using the formula $W_i = [(W_i - W_d)/ W_d] \times 100$, where W_i and W_d are the masses of imbibed and dry tissues, respectively.

Endocarp and Seed Coat Anatomy

Mature fruits (*c.* 397 DAF) had the exo- and mesocarp removed by scraping with a knife to expose the endocarp. Once the outer fruit tissues were removed, endocarps were cracked with pliers to remove seeds. Seeds were trimmed further with horticultural pruners and the rotary tool until sections approximately 5 x 5 x 2 mm remained.

Endocarp and seed coat pieces were stored at 4°C in DMSO until sectioning. Tissues were mounted on stubs with freezing media and sectioned on a Leica CM 1850 freezing microtome. Sections ranged from 10 to 60 μ and were stained with toluidine blue or phloroglucinol to visualize lignins or pectic substances. Sections were viewed at 6.3 to 63 x on a Zeiss compound microscope and photographed with a Nikon Cool-pix digital camera.

Mechanical Restraint by Covering Structures

Germination of Seeds with the Pericarp Removed

Seeds were extracted from fruits and dusted with a fungicide (Dimethyl 4, 4'-0-phenylenebis-3-thioallophanate). The dusted seeds were then sown as described for the move-along experiments. Germination counts occurred 7 d after sowing and then every 12 d for 67 d. Seeds were counted as germinated when the radicle had extended $\geq$ 1mm from the emerged cotyledonary stalk.

Germination of Seeds in Light or Darkness

To determine if light is a requirement for germination seeds were sown, as described previously, in trays that were covered in two layers of aluminum foil or left uncovered. Trays were placed in an incubator set to constant 35°C with a 14 h photoperiod for 28 d. After this period, trays were removed and the number of germinated seeds was counted.

Embryo Emergence from Seeds with Opercula Intact or Removed

The purpose of this experiment was to determine if the operculum delays embryo emergence and therefore subsequent radicle protrusion. Fruits were harvested at shedding, the seeds were extracted and dusted the seeds with fungicide. After dusting,

five replications of 10 seeds each had the operculum removed with a knife or were left intact. Seeds were sown at constant 35°C with an alternating 14 h photoperiod. Germination was checked at weekly intervals for four weeks. Germination, in this case, was counted when the operculum was displaced from the seed coat by ≥ 1 mm or when the embryo had extended past the seed coat by ≥ 1 mm in seeds with no operculum.

Effect of GA_3 on Operculum Displacement

Four replicates of 10 seeds each were soaked in 0, 10, 100 or 1000 ppm GA_3 (United States Biochemical Corp., Cleveland, OH) solutions for 24 h. After soaking seeds were rinsed with distilled water for 1 min and sown at 35°C with a 14 h alternating photoperiod. Germination was checked at weekly intervals for four weeks. A seed had germinated when the operculum was displaced ≥ 1 mm.

Embryo Growth

Accessible inflorescences were tagged at anthesis from one tree, growing on university grounds and 10 fruits harvested at approximately 30 d intervals throughout development. At each harvest date the length of excised embryos was measured with digital calipers. Fruits were also collected at shedding from multiple trees, the seeds were extracted, and their length measured. After being measured, seeds were dusted with fungicide and placed in trays within an incubator set to constant 35° C with an alternating 14 h photoperiod. The length of embryos was then measured when they displaced the operculum from the seed coat by ≥ 1 mm or when radicles protruded from the cotyledonary stalk by ≥ 1 mm. The water content of embryos at shedding, operculum displacement, and radicle protrusion was determined as well.

The water potential of embryos was calculated at these stages of development by constructing isotherms. One embryo replicated five times was soaked in polyethylene glycol (PEG, M_r = 8000) solutions of different concentrations ($\Psi_w \geq$ -12 MPa) for 48 h at 25°C. The embryos were blotted dry after soaking, and then the fresh and dry mass of each embryo was recorded. Water content was determined as above. The water potential of different PEG solutions at different temperatures was calculated according to Michel and Kaufman (1973) and verified by thermocouple psychrometry (Model SC-10, Decagon Devices, Pullman, WA, USA). The water content and water potential of developing embryos was related with the water sorption isotherms.

Statistical Analysis

Where appropriate, the final germination percentages for all treatments was recorded and mean time to complete germination (MTG) was calculated using the formula: MTG = $\Sigma (f \cdot x)/ \Sigma f$; where f is the number of seeds germinated on day x and x is the number of days after sowing. Additionally, the final water content and final fresh mass for the endocarp imbibition and mechanical scarification experiments were calculated, respectively. These data were analyzed using analysis of variance (ANOVA). Main effects were separated using Duncan's Multiple Range (DMR) test. Data based on percentages were arcsin transformed. Conclusions did not change due to transformations, therefore, analyses of untransformed data are presented.

Linear regression was used to analyze the relationships between embryo length, moisture content, and water potential. These data were transformed to meet the assumptions of ANOVA. All data were analyzed using SAS version 9.1 (SAS Institute Inc., Cary, NC) in PROC GLM or PROC REG.

Results

'Move-Along' Experiments

Heavy fungal contamination was apparent on epicarps until about 4 weeks after sowing and intact fruits did not germinate at any test condition for the first 5 weeks of incubation (Fig. 4.2). Percent germination increased between 6 and 13 weeks and maximum germination in the alternating temperature regimes was achieved after 15 weeks of incubation. At 15 weeks of incubation germination of fruits held at simulated environmental temperatures reached 12 to 20%. However, the germination percentage of fruits held at constant 25°C was double that of fruits at alternating temperatures. Temperature did not affect germination kinetics, but did affect final germination percent. Fruits incubated at 35° or 15°C did not germinate during this time interval (Fig. 4.2).

After 13 weeks of incubation fruits at the alternating temperatures were moved to the next test conditions. A shift in incubation temperature did not cause an increase in germination. However, slight improvement in germination was observed for fruits held at 25°C 21 weeks after sowing. A slight increase in germination was observed at 26 weeks after sowing for drupes at alternating temperatures.

Imbibition Studies

Imbibition of Excised Embryos, Seeds, and Endocarps

The fresh mass of excised embryos increased by 73% over the first seven hours of imbibition and remained relatively stable until 72 h. At 24 h of imbibition the fresh mass of embryos decreased slightly (Fig. 4.3), despite their fully turgid appearance. The decrease was not significantly different ($F = 0.74$, $P = 0.40$). Droplets of an unknown liquid were exuded from the surface of embryos not in contact with the moist blotter at

this time. Fresh mass decreased again at 96 h of imbibition, but increased to 93.1 ± 9.5% when the experiment was terminated at 168 h (Fig. 4.3). Some embryos turned gray and became flaccid during this interval. Embryos that had significantly deteriorated were removed and the fresh mass adjusted accordingly. Radicle protrusion did not occur in the remaining turgid embryos at any time during this trial.

Embryos excised from seeds that had imbibed for 336 h increased in fresh mass by 127.2 ± 11.8 %. If endocarps were present then embryos increased in fresh mass by 93.1 ± 5.3% after 336 h of imbibition (Fig. 4.3). Although a few embryos had imbibed sufficiently to displace the operculum from seeds at 336 h, no opercula were displaced from seeds within endocarps. None of the embryos that dislodged opercula in this experiment showed radicle protrusion. Embryos in tissue culture produce radicles in 7-14 d (Pérez, unpublished results).

Imbibition occurred slowly in intact seeds or endocarps (Fig. 4.3). Seeds increased in fresh mass by 1.8 ± 0.23% at 1 h of imbibition. Similarly, imbibition of endocarps reached 1.1 ± 0.11% at 1 h. After 24 h of imbibition seeds or endocarps increased in fresh mass to 6.3 ± 0.58% and 5.3 ± 0.31%, respectively. The fresh mass of seeds and endocarps at 336 h of imbibition were 18.4 and 15.9% greater than initial values, respectively (Fig. 4.3). A few opercula were dislodged from seeds; however, no radicle protrusion took place.

Effect of Endocarp on Imbibition

Table 4.1 shows mean moisture content and standard error of embryos, seeds and isolated endocarps after zero and four weeks of imbibition in the presence or absence of endocarps. The moisture content of embryos, seeds and endocarps is low at the start of

the experiment. However, moisture content increases greatly after four weeks of imbibition regardless of the presence or absence of endocarps.

The presence or absence of endocarps did not significantly affect the ability of embryos or seeds to imbibe ($F = 3.62$, $P = 0.09$). However, a significant increase in the moisture content of embryos, seeds, and isolated endocarps occurred after four weeks of imbibition ($F = 578.50$, $P < 0.0001$). There was no interaction between the level of endocarp remaining or time ($F = 2.55$, $P = 0.14$).

Mechanical Scarification Effects on Imbibition of Seeds and Endocarps

After 24 h of imbibition the fresh mass of seeds scarified for 3 or 5 s increased by 9.9 ± 0.15% and 8.2 ± 0.37%, respectively. Control seeds increased in fresh mass by 8.8 ± 0.37% at the same time interval. The increase in fresh mass of seeds between 24 and 336 h ranged from 23.6 to 29.6% (Fig. 4.4). Fresh mass was lower in scarified or non-scarified endocarps than seeds at 24 h. Scarified endocarps increased in fresh mass by 5.32 ± 0.24 %, while controls increased by 5.05 ± 0.24 %. Regardless of the treatment, seeds imbibed more rapidly than endocarps throughout the experiment, especially from 24 h onward. On the other hand, the increase in fresh weight for endocarps never exceeded 16.0 ± 1.02 % (Fig. 4.4).

The hypotheses that mechanical scarification increases the ability of seeds or endocarps to imbibe is rejected. Analysis of variance has shown that no differences exist ($F = 1.31$, $P = 0.34$) among seeds left un-scarified or scarified for 3 or 5 s. Similarly, there is no evidence ($F = 2.55$, $P = 0.21$) to suggest that scarified endocarps increase in fresh weight more than non-scarified endocarps.

Endocarp and Seed Coat Anatomy

The endocarp of mature *P. remota* fruits (*c.* 397 DAF) is composed of an outer, middle, an inner layer (Fig. 4.5). The outer and inner layers have files of tangentially stretched cells with some lignin deposits (Fig. 4.5). The middle layer is thicker and composed of thick-walled, pitted, sclereids. Lignin deposits are found throughout the middle layer, but accumulated towards the inner endocarp layer.

The seed coat is several cell layers thick, but these cells are not arranged as a palisade layer (Fig. 4.6). The seed coat is heavily lignified as evidenced by phloroglucinol and toluidene blue staining. Notably, a heavy deposition of lignin is noticeable where the inner integument and endosperm meet (Fig. 4.6). The operculum is subtended by 1-2 layers of endosperm towards the center (Fig. 4.6), but a thicker layer of lateral endosperm forms where the embryo cavity gives way to the thick-walled endosperm (Fig. 4.6).

Mechanical Restraint by Covering Structures

Germination of Seeds with the Pericarp Removed

Germination of seeds was faster and more complete when the covering fruit structures were removed. For example, at 31 d post-sowing seeds held at 35°, 25°, 26/19.5°, and 28.5/22°C germinated to 98, 81, 68, and 54%, respectively. Germination was 90% or greater for these treatments by 67 d after sowing. However, seeds held at 15°C germinated to 22% by this day (Fig. 4.7). The MTG of seeds incubated at various temperatures is shown in Table 4.2.

The hypothesis that treatments differed in their effect is supported. All temperature treatments significantly affected the final germination percentage of *P.*

remota seeds ($F = 102.01$, $P = < 0.0001$). The final germination percent between seeds exposed to constant warm or alternating temperatures were similar according to DMR test, but these values differed from final germination at 15°C (Table 4.2). Additionally, the temperatures to which seeds were exposed significantly affected MTG ($F = 499.90$, $P < 0.0001$). According to DMR test, four homogeneous groups of MTG were identified (Table 4.2).

Seeds incubated in alternating light regimes or darkness germinated to 90 ± 4 % or 91 ± 5 % (mean ± SE), respectively, at 28 d. The hypothesis that no differences in germination occur for seeds incubated in light or dark is supported ($F = 0.02$, $P = 0.91$).

Embryo Emergence from Seeds with Opercula Intact or Removed

After seven days of incubation at 35°C 84% of seeds with no opercula had embryos that extended ≥ 1 mm past the seed coat. All seeds in this treatment germinated by day 14. The MTG for seeds with no opercula was 8.1 ± 0.36 d. Alternatively, only 8% of control seeds had embryos that displaced the opercula by ≥ 1 mm after seven days of incubation. At 28 d of incubation 98.0 ± 4% of control seeds had dislodged the operculum. It took 15.8 ± 0.59 d on average for seeds with intact opercula to complete germination (Fig. 4.8). The evidence supports the hypothesis that de-operculation accelerates germination. Analysis of variance identified a highly significant difference of MTG ($F = 110.79$, $P = 0.0005$) between the two treatments.

Effect of GA_3 on Operculum Displacement

Figure 4.9 displays the mean percent germination of seeds after treatment with or without GA_3. Germination of treated seeds was 23 to 39% greater on average than non-treated seeds, with larger differences in germination being apparent earlier in the trial.

Final germination ranged between 97.5 and 100% for treated seeds, while controls germinated to 75% at the end of the experiment.

There is sufficient evidence to reject the hypothesis that final germination percentages are equal among different GA treatments. For instance, treatment differences were significant ($F = 11.00$, $P = 0.002$). Duncan's Multiple Range test declared the mean differences in germination of treated seeds to be the same, but these means were significantly different than controls at the $P < 0.05$ level. On the other hand GA_3 treatment made no difference in MTG ($F = 2.71$, $P = 0.10$).

Embryo Growth

Embryos increased from about 1 to 5 mm in length between 100 and 250 days after flowering (DAF). At 250 DAF embryos have attained maximum dry weight (see Chapter 2). Embryos decreased in length during the latter stages of development and were shed at 4.23 ± 0.06 mm in length (Fig. 4.10).

Embryos, from seeds of several trees, were 3.92 ± 0.07 mm in length and their moisture content was 0.39 ± 0.04 g g^{-1} at shedding (Fig. 4.11). The mean length of seeds was 13.30 ± 0.19 mm; therefore, embryos were 29.5% the length of seeds. Embryos must increase in size by 58.7% in order to displace opercula (Fig 4.11). This increase in embryo length correlated with a 746.2% ($R = 0.98$) increase in moisture content and a 1.5 x 10^5 increase (*i.e.* less negative) in water potential ($R = 0.99$) (Fig 4.12). Radicle protrusion did not occur upon operculum displacement. Instead, embryos elongated by 166.8% and increased in moisture content by 1379.5%, from initial values at shedding, before radical protrusion occurred (Fig. 4.11). The water potential of embryos had increased by 1.1 x 10^6 at radicle protrusion (Fig. 4.12).

The hypothesis that embryos reach a critical length, moisture content, and water potential before operculum displacement and radicle protrusion occur is supported. Regression analysis revealed that the relationship between increasing embryo length and moisture content was highly significant. Likewise, the hypothesis that water potential had no effect on embryo length can be rejected (Table 4.3).

Discussion

Mature, intact drupes of *P. remota* failed to complete germination over a range of temperatures and are considered dormant at shedding (*sensu* Baskin and Baskin 2001). Radicle protrusion in drupes incubated at simulated alternating environmental temperatures was first observed 6 weeks after sowing. Drupes continued to complete germination sporadically over the next 20 weeks under these conditions. Radicle protrusion was delayed further when drupes were incubated at constant 25°C, but eventually germination at this temperature surpassed that at other temperatures. Similarly, *P. remota* fruits sown outdoors in Manoa and either partially buried to their midline or buried 5 cm had 2% germination 5 weeks after sowing. *P. remota* fruits maintained on the soil surface did not germinate for the duration of these trials (*i.e.* 30 weeks) (Pérez, unpublished data). Fruits of the related species, *P. hillebrandii* and *P. kaalae*, sown in a shadehouse commenced germination around 9 weeks after sowing if partially buried and at 13 weeks if buried (Pérez, unpublished data). It is common for palm seeds to begin germinating within 17 weeks and to show a period of dormancy (Orozco-Segovia *et al.* 2003).

Baskin and Baskin (2001) have outlined several types of dormancy and their underlying mechanisms. These include physiological dormancy, which among other

factors, may be due to embryo covering structures, and morphological dormancy. Seeds that are considered morphologically dormant have undifferentiated or underdeveloped embryos that must continue to grow and reach a critical size before radicle protrusion takes place. In *P. remota*, embryo growth is restricted by the operculum and endocarp. Secondly, embryos are underdeveloped and must reach a critical length, moisture content, and water potential for operculum displacement and subsequent radicle protrusion. *Pritchardia remota* drupes and seeds are capable of imbibition and are therefore not physically dormant (Baskin and Baskin 2001). Germination is stimulated by warm-constant temperatures; therefore seeds may be classified as possessing non-deep physiological dormancy in addition to morphological dormancy. The physiological dormancy mechanisms must be overcome first, before morphological dormancy is alleviated.

Temperature is perhaps the critical factor in alleviating dormancy and promoting germination (Vleeshouwers *et al.* 1995; Probert 2000). Generally, warm temperatures (30-40°C) are necessary to break dormancy and promote germination in palms (Broschat and Meerow 2000; Orozco-Segovia *et al.* 2003). Although optimum temperatures for dormancy break and germination were not determined for *P. remota* drupes, clearly temperatures $\geq$35°C or $\leq$ 15°C are outside the optimum range for this species. In contrast, *E. guineensis* has evolved dormancy-breaking and germination-promoting mechanisms for high temperatures in its home range (Hussey 1958; Rees 1962). Although germination is first observed in drupes held at alternating temperatures, those at constant 25°C eventually began to germinate more rapidly and to a higher percent (see Fig. 4.2).

Generally, palm seeds have a narrow temperature range, usually 5-10°C, around the optimum that allows germination to be completed. This seems to be the case for *P. remota*, where dormancy is alleviated and germination stimulated by temperatures about 25°C. Temperatures at sea level in Hawaii are mild all year. Although some day-to-day variation exists between day and night air temperatures, seasonal differences are minor. For instance, average maximum air temperatures at Lihue, Kauai (the location of an official monitoring station closest to Nihoa) are between 29 to 26°C. Average minimum air temperatures throughout the year are 22 to 26°C. The soil temperatures, where germination occurs, are not known.

Currently, primary dispersal of *Pritchardia* fruits is by gravity and the majority of fruits, if not eaten by predators, are found near the parent tree (Pérez, pers. obs.). Air and soil temperatures under a *Pritchardia* canopy are also cooler than exposed sites (Pérez, unpublished data). Therefore it is reasonable that *Pritchardia* has evolved mechanisms for the alleviation of dormancy and promotion of germination at temperatures near 25°C.

The endocarp and seed coat of *P. remota* are permeable, although imbibition is slow. None of the covering structures of *Jubaeopsis caffra* Becc. (Arecaceae) seeds block imbibition (Robertson and Small 1977). Regardless of the covering structures in *P. remota*, embryos become hydrated more rapidly and to higher moisture content than the remaining seed tissue. Likewise, the endosperm of maize (McDonald *et al.* 1994) and barley (Allen *et al.* 2000) lag in imbibition compared to the embryo. Endosperm cell walls in the palms *P. dactylifera* and *Washingtonia robusta* H. Wendl. comprise 49 and 65% of total cell volume and are composed primarily of β-(1, 4)-mannose (DeMason

1985). It is likely that the slow uptake of water in *P. remota* seeds is a result of its dense endosperm.

Pritchardia remota endocarps are multi-layered and possess sclereids with substantial lignin deposits. Similarly, seeds coats are lignified and possess a distinct lignin layer at the testa-endosperm interface. However, no palisade layers of thick-walled cells were detected in endocarps or seeds. Therefore limited similarities to seeds with impermeable coats are apparent (Li *et al.* 1999, 1999; Baskin and Baskin 2001; Meisert 2002). For this reason, mechanical scarification may not improve imbibition in endocarps or seeds compared to non-scarified controls. This runs contrary to the idea that imbibition is blocked by the fruit or seed coats of palms and emphasizes the need for imbibition and anatomical studies to determine where water does enter the seed.

At shedding the pericarp of *P. remota* consists of a thin exocarp, fibrous mesocarp, and relatively thick, hard endocarp. Removal of the pericarp vastly increased uniformity and speed of germination. Clearly the pericarp, more specifically the endocarp, offers mechanical resistance to the elongating embryo. Cleaning palm seeds (*i.e.* removing the entire fruit coat) results in significantly faster and more complete germination in variety of palms (Broschat and Donselman 1986; Orozco-Segovia *et al.* 2003; Briceño and Maciel 2004), including *Pritchardia* (Yoshii and Rauch 1989; Rauch 1994; Maciel and Mogollón 1995).

Warm constant temperatures (25 and 35°C) are more effective than alternating temperatures at promoting germination in cleaned *P. remota* seeds. Seeds of *S. palmetto* germinated to higher percentages at constant rather than alternating temperatures (Brown 1976). Germination of *Thrinax morrisii* H. Wendl. and *Coccothrinax argentata* (Jacq.)

L. H. Bailey was more rapid at constant temperatures; however, more seeds germinated at alternating temperatures (Carpenter 1988).

It should be noted that *P. remota* seedlings held at 35°C from seeds that germinated at the same temperature were much less vigorous than those for seeds germinated and grown at alternating temperatures or constant 25°C (Pérez, unpublished results). A similar response was observed for *Butia capitata* (Mart.) Becc. (Carpenter 1988). Pre-treatment at 35°C may serve as a suitable dormancy breaking treatment, if seeds are then returned to cooler temperatures for germination and seedling growth. *Elaeis guineensis* seeds are handled much the same way (Hussey 1958).

In addition, light may (Wood and Pritchard 2003) or may not (Brown 1976) be a requirement for germination of some palms. Seeds of *P. remota* do not require light for germination. Similarly, seeds of *P. hillebrandii* and *P. kaalae* germinated to high proportions in light or darkness (Pérez, unpublished data). The lack of a light requirement is reasonable as *Pritchardia* fruits often become buried by minor landslides and dead leaves (Pérez, pers. obs.) or vertebrate activity (David Woodside, pers. comm.).

The operculum, in addition to the endocarp, significantly delays emergence of the embryo, thus creating another layer of mechanical restraint. The operculum of *E. guineensis* forms a mechanical barrier to embryo elongation. Only when embryo dormancy has ceased and the middle lamella of operculum cells breaks down does the operculum abscission zone function, allowing embryo emergence (Hussey 1958). Coat-imposed dormancy in *R. hystrix* is caused by the sclerotesta and operculum. Germination is significantly enhanced only after these tissues are removed (Carpenter and Cornell 1993).

Gibberellins can break physiological dormancy and stimulate germination in many species (Baskin and Baskin 2001). What's more, gibberellins have been implicated in the stimulation of cell-wall degrading enzymes that may reduce mechanical resistance of covering structures (Groot and Karssen 1987; Groot *et al.* 1988; Bewley 1997; Ren and Kermode 2000). Cell wall degrading enzymes have been identified for some palms (DeMason 1985). In palms the role of gibberellins is equivocal (Nagao and Sakai 1979; Yoshii and Rauch 1989). For *P. remota* the speed of germination was not significantly affected by gibberellin application, but final germination percent increased over non-treated seeds. It is plausible that GA_3 stimulated the production of enzymes that degraded the walls or middle lamella of endosperm cells subtending the operculum, thereby reducing the mechanical resistance of this structure. Alternatively, Gong *et al.* (2005) conclude that "germination" in *P. dactylifera* is related to operculum anatomy rather than specific enzyme activity.

Often, extension of the cotyledonary stalk is referred to as germination, giving the impression of radicle protrusion and the completion of germination. This is not the case. Palm embryos are underdeveloped at shedding (Fong 1978; Uhl and Dransfield 1987; Ehara *et al.* 1998; Wood and Pritchard 2003). Due to the density of endosperm no room exists within palm seeds for complete embryo development. Thus for radicle protrusion and seedling growth to occur, the embryo must extricate the shoot and root meristems. This form of germination is an adaptation to climatic conditions (Uhl and Dransfield 1987).

The underdeveloped embryos of *P. remota* must reach a critical length, moisture content, and water potential before operculum displacement and radicle protrusion occur.

Germination events in *H. lagenicaulis* seeds also occur at critical water contents (Wood and Pritchard 2003). Consequently, operculum displacement, by the extending embryo, should be considered as one step along the germination continuum. In fact, it may take 7 to 14 d between operculum displacement and radicle protrusion in *Pritchardia* (Hodel 1977); Pérez pers. obs.). Embryo growth leading to operculum displacement could be a combination of cell elongation and division due to substantive increases in water content and water potential (*i.e.* less negative) (Myers *et al.* 1992; Vertucci and Farrant 1995).

Dormancy-break in *P. remota* seems to be facilitated by constant warm temperatures (see Fig 4.2). For example, at 35°C, O_2 uptake in *P. remota* embryos is greater compared to embryos at 25° or 15°C (see Chapter 5). Therefore, embryos may be producing the necessary products for alleviation of dormancy and germination at a faster rate. Imbibition is known to be influenced by temperature (Vertucci and Leopold 1983) among other factors. It is plausible that *P. remota* seeds may imbibe more quickly at higher temperatures, resulting in earlier germination. Seeds may also surpass their hydrothermal threshold (Bradford 1990, 1995, 2002) sooner at 35°C. The total lipid fraction of *P. remota* embryos is composed of 42% saturated and 58% unsaturated fatty acids (Pérez and Walters unpublished data). Warmer temperatures may be necessary to melt energy-rich saturated fatty acids thereby permitting imbibition and germination (Linder 2000; Crane *et al.* 2003). It is unlikely, however, that only one of these factors is responsible for dormancy break and the completion of germination.

The adaptive significance of slow imbibition, multiple restraint mechanisms, and underdeveloped embryos is to ensure that germination is completed when the chances for seedling survival are at or near optimal. However, it has become increasingly difficult

for the majority of *Pritchardia* species to carry out this natural process in the wild. For example, non-native feral ungulates and rodents decimate seeds and seedlings (Arcand *et al.* 2003). On Nihoa, the invasive grasshopper, *Schistocera nitans* has caused serious damage to *P. remota* through defoliation and consumption of flowers and developing fruits (Alex Wegmann, U.S.F.W.S, pers. comm.). Therefore, understanding the multiple dormancy mechanisms in *P. remota* may assist in meeting *in situ* and *ex situ* recovery objectives.

Tables

Table 4.1. Changes in moisture content of embryos (isolated from seeds), seeds (with embryos removed), and endocarps (with seeds removed) after zero and four weeks of imbibition in 35 mL of water at ~ 23° C. Interactions were not significant. Mean separation was by Duncan's Multiple Range Test. Numbers within a column followed by the same letter are not significantly different ($\alpha = 0.05$)

	Moisture Content ($g\ g^{-1}$)		
Treatment	Embryo ± SE	Seed ± SE	Endocarp ± SE
- endocarp 0wk	0.36 ± 0.05^a	0.24 ± 0.03^a	-
+ endocarp 0wk	0.34 ± 0.05^a	0.28 ± 0.03^a	0.15 ± 0.01^a
- endocarp 4wk	2.05 ± 0.06^b	0.57 ± 0.01^b	-
+ endocarp 4wk	1.82 ± 0.10^b	0.56 ± 0.03^b	0.33 ± 0.01^b

Table 4.2. The mean time to complete germination (MTG) in days and final germination (FG) percent of *P. remota* seeds incubated at constant or alternating temperatures for 67 days. Numbers within a column followed by the same letter are not significantly different at $\alpha = 0.05$. Means separated using Duncan's Multiple Range Test. Arcsin transformation of germination data did not result in different conclusions, therefore original values are shown. The coefficient of variation for MTG and FG are 4.4 and 9.1, respectively.

Treatment (°C)	MTG ± SE	FG ± SE
35	22.4 ± 0.9^a	100.0 ± 0.0^a
28.5/22	36.6 ± 0.9^c	90.0 ± 3.2^a
26/19.5	34.5 ± 0.8^c	97.8 ± 2.2^a
25	30.8 ± 1.0^b	100.0 ± 0.0^a
15	67.0 ± 0.0^d	22.4 ± 5.8^b

Table 4.3. ANOVA tables for regression analysis of embryo length and moisture content

Source	df	Mean Square	*F*	*P*
Regression	1	1.89	263.36	<0.0001
Error	10	0.01		
Corrected Total	11			

Figures

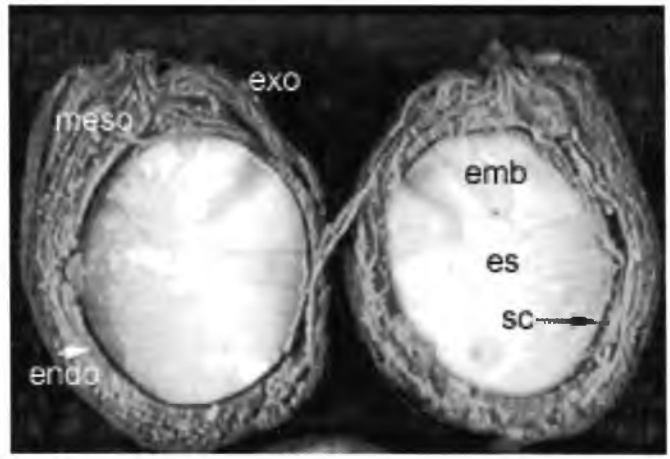

Fig. 4.1. Cross section of freshly shed *Pritchardia remota* fruit and seed. Exo = exocarp, meso = mesocarp, Endo = endocarp, SC = seed coat, ES = Endosperm, Emb = embryo

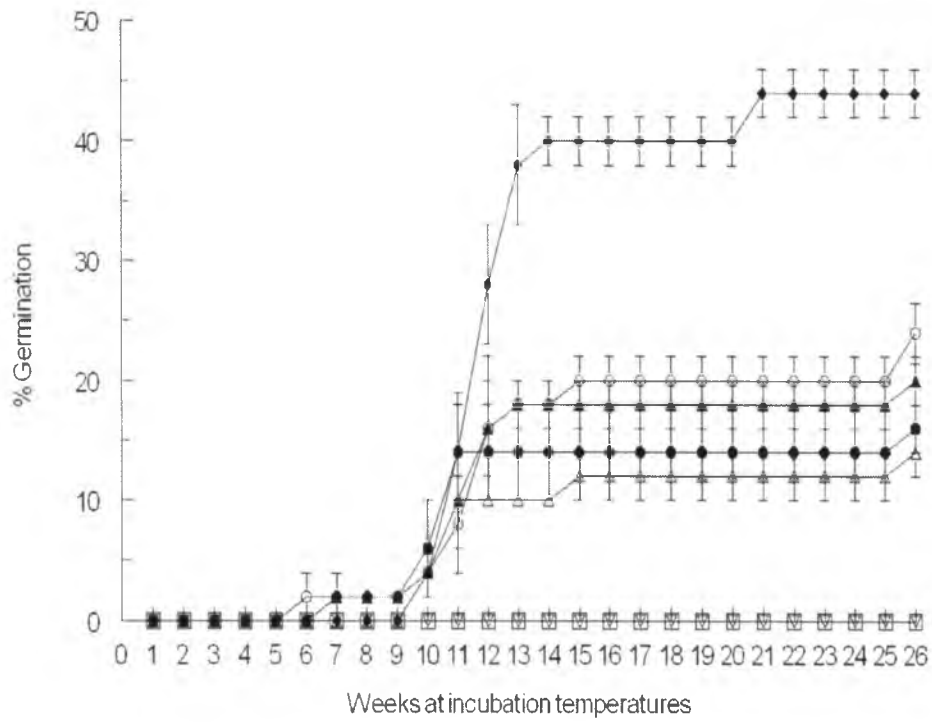

Fig. 4.2. Emergence of *Pritchardia remota* radicles through the pericarp after sowing at 35°C (open squares); 28.5/22°C (triangles); 26/19.5°C (circles); 25°C (diamonds); and 15°C (open, inverted triangles). Solid triangles and circles denote trays that were moved after 13 weeks, while open triangles and circles remained at the respective temperatures for the duration of the experiment. Error bars denote SE.

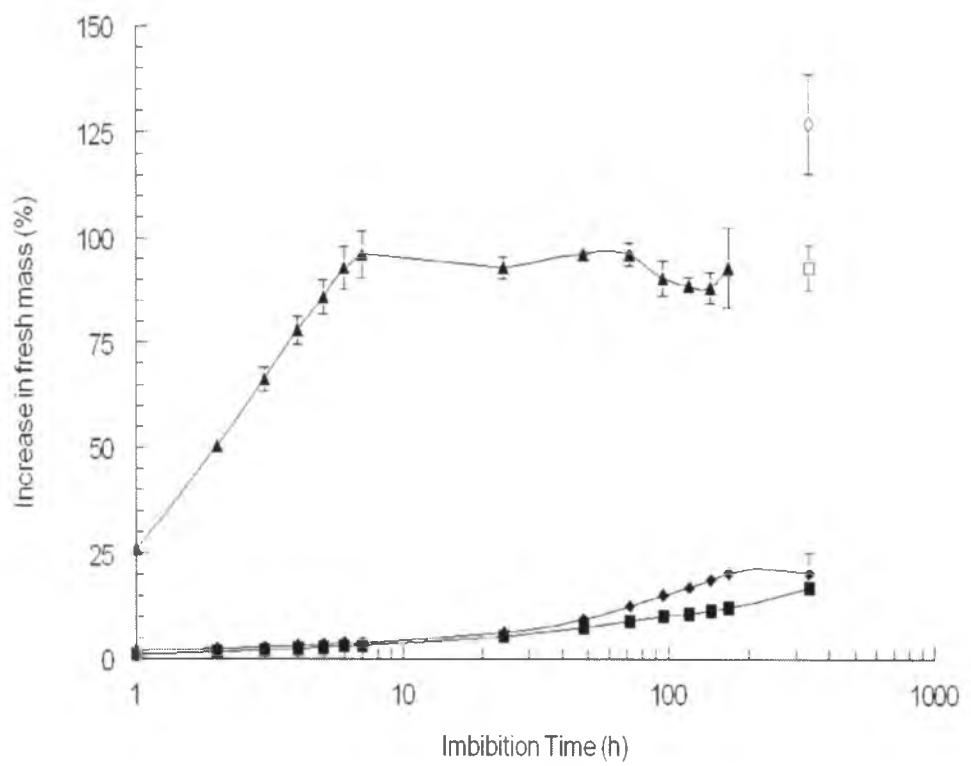

Fig. 4.3. A comparison between the increase in fresh mass of excised *Pritchardia remota* embryos placed on moist blotter (triangles) and the increase in fresh mass of seeds without pericarp (*i.e.* exo-, meso- and endocarp) tissues (diamonds) and endocarps with the exo- and mesocarps removed (solid squares) after imbibition in 30 ml water. Fresh mass was also taken after 336 h for embryos excised from seeds (open diamonds) and endocarps (open squares). Error bars signify SE.

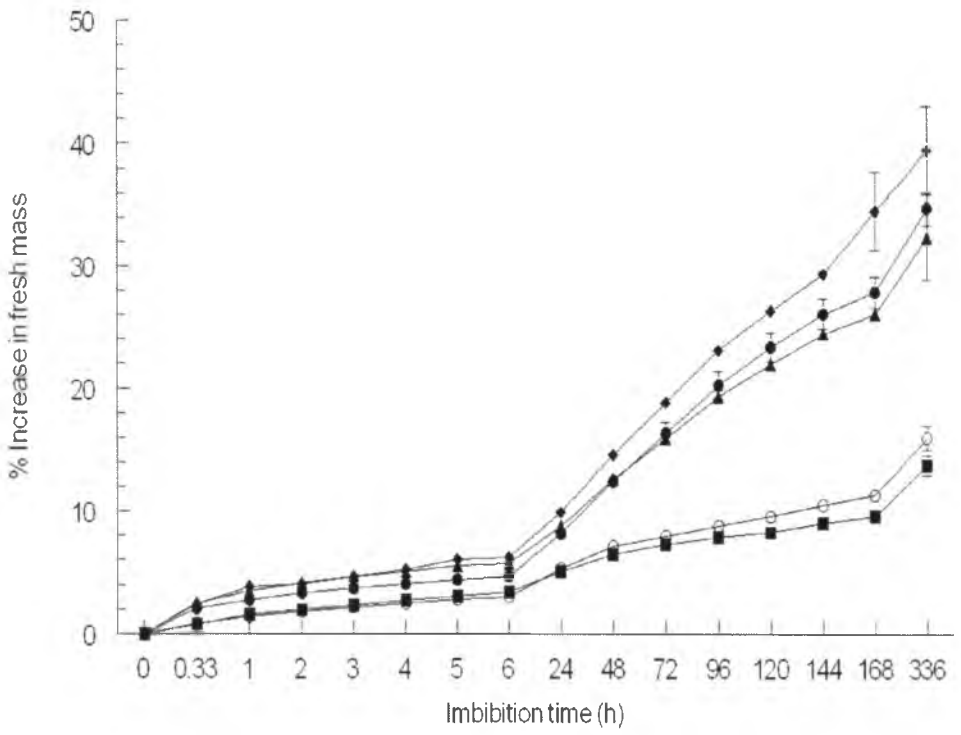

Fig. 4.4. Increase in fresh mass for seeds left un-scarified (triangles), scarified for 5 sec. on the seed coat (closed circles), or scarified on the operculum for 3 sec. (diamonds), and intact endocarps (squares) or endocarps scarified for 5 sec. (open circles). Scarification was with a mechanical sanding device. Error bars show SE.

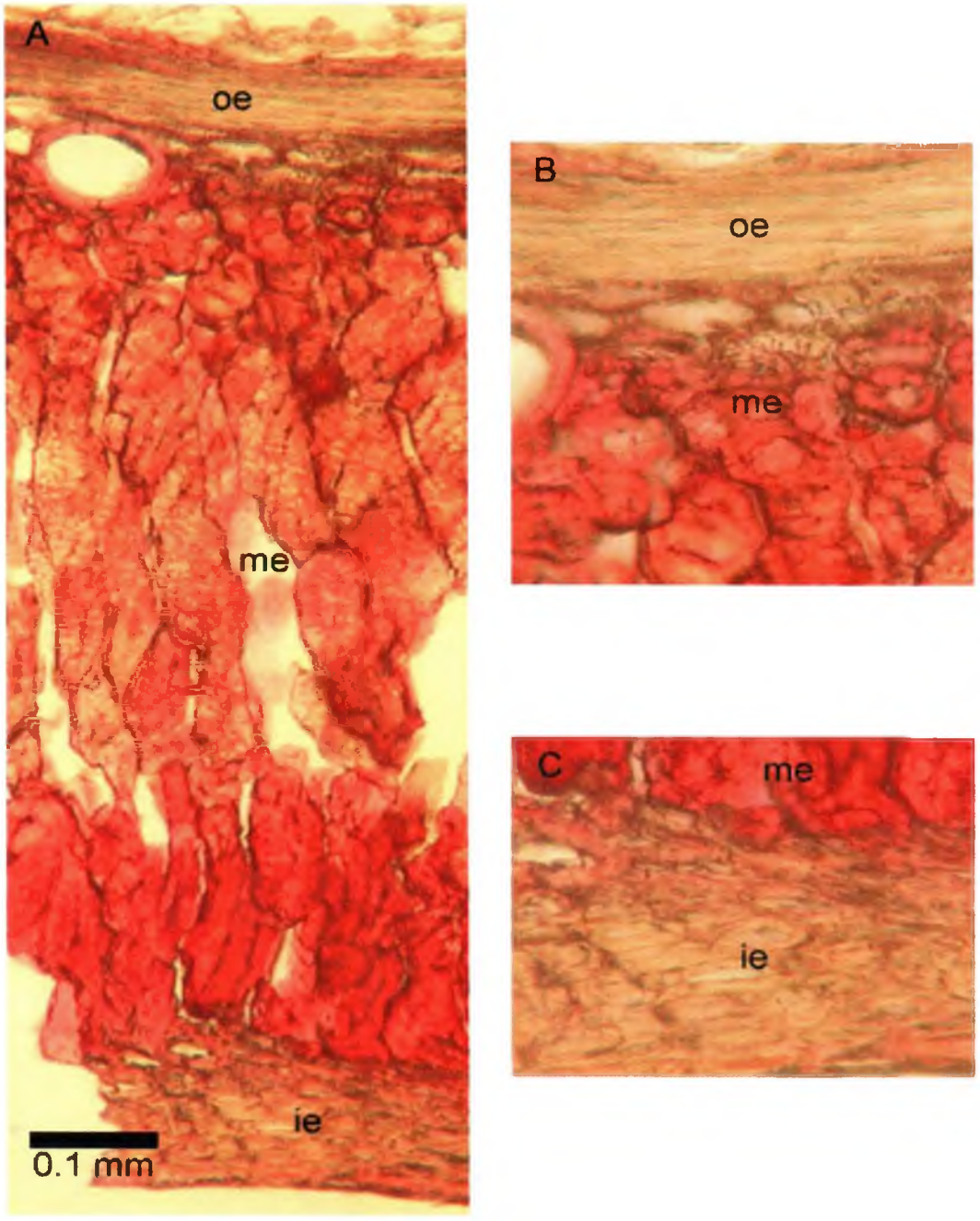

Fig. 4.5. Anatomy of mature *Pritchardia remota* endocarps. Note the multiple layers (A) comprising the endocarp. The middle endocarp (ME) is composed of large sclerids with lignin deposits. (B) and (C) are details of the outer (OE) and inner (IE) endocarp repectively. Staining was by phloroglucinol. (A) 6.3 x, (B) and (C) 16 x. All sections 30μ.

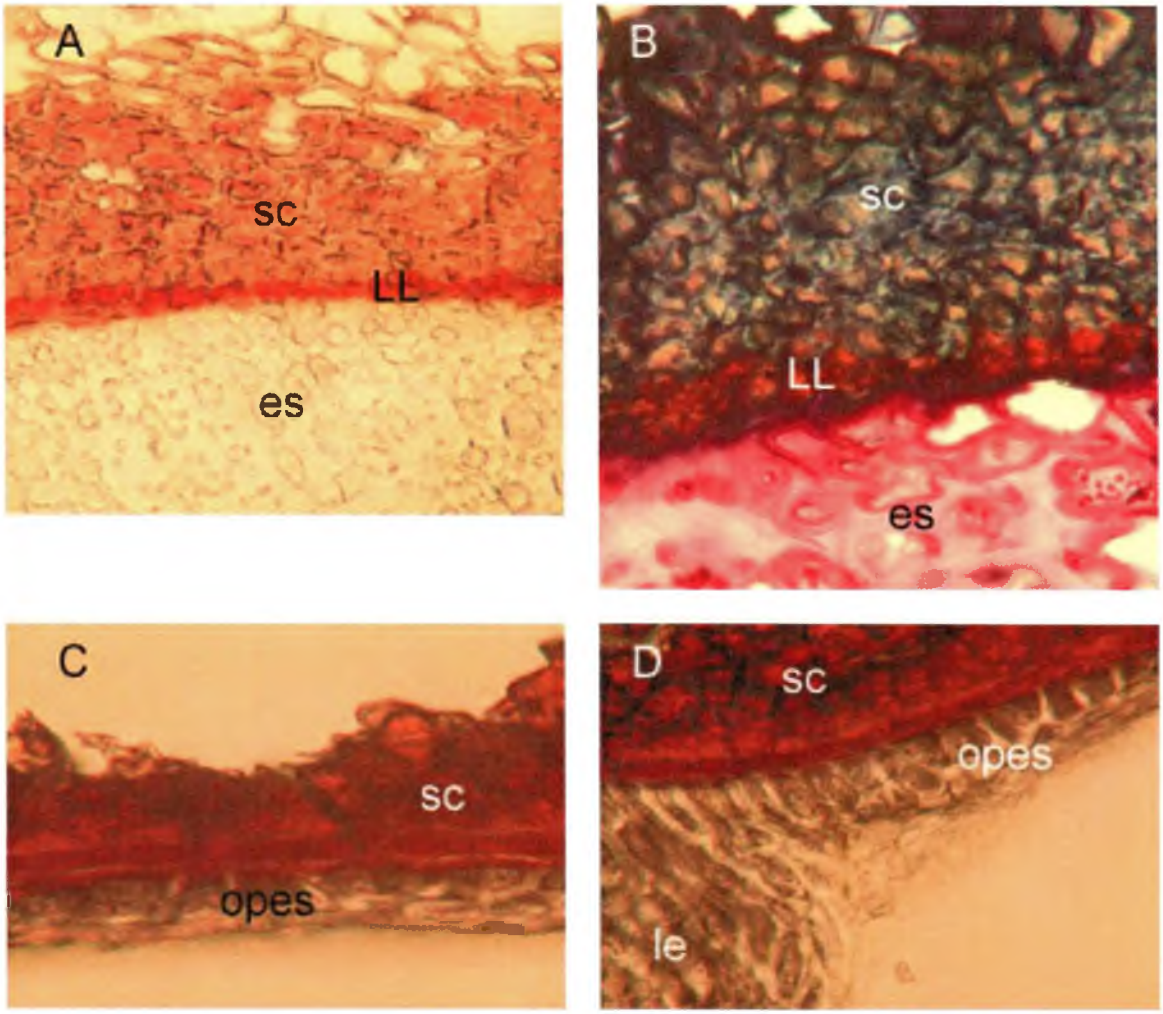

Fig. 4.6. *Pritchardia remota* seed coat and operculum anatomy. Seed coats (sc) stained with phloroglucinol (A) or toluidene blue (B) display lignification of the cells and a prominent lignin layer (LL). The thick cell walls of the endosperm (es) are rich in polysaccharides as evidenced by Tolouidene Blue staining. Seed coat sections 10μ. Thick sections (50μ) of the central (C) and lateral (D) portions of opercula. Note that the central part of the operculum is subtended by a layer of endosperm 1-2 cell layers thick, while lateral portion have thicker underlying endosperm layers. Opes = operculum endosperm, le = lateral endosperm. (A) and (B) 6.3x. (C) and (D) 16x.

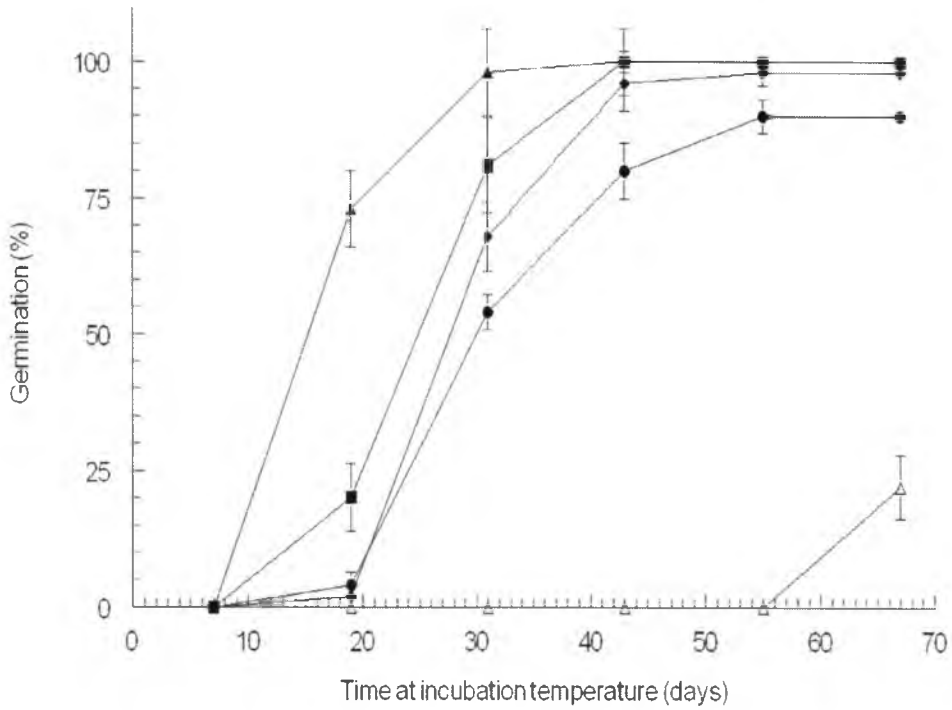

Fig. 4.7. Germination (*i.e.* radicle protrusion) of *Pritchardia remota* seeds incubated at 35° (closed triangles), 28.5/22° (circles), 26/19.5° (diamonds), 25° (squares), and 15° (open triangles) C. Seeds were removed from fruits prior to incubation. Error bars denote SE.

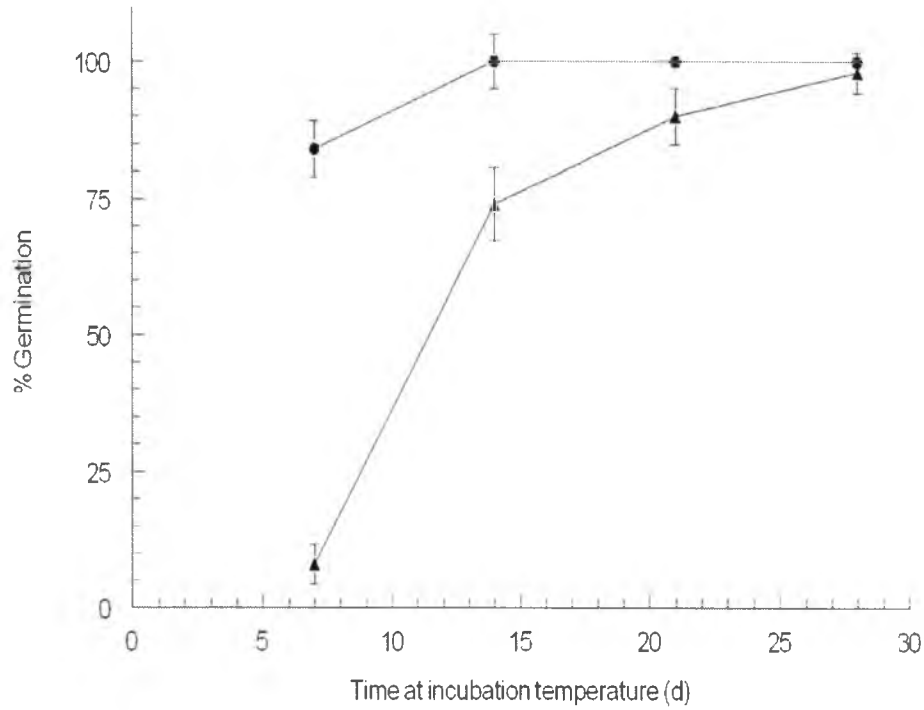

Fig. 4.8. Percentage of *Pritchardia remota* seeds with the operculum removed and embryos protruding 1mm or greater past the seed coat (circles) or with intact opercula (triangles). Error bars are SE.

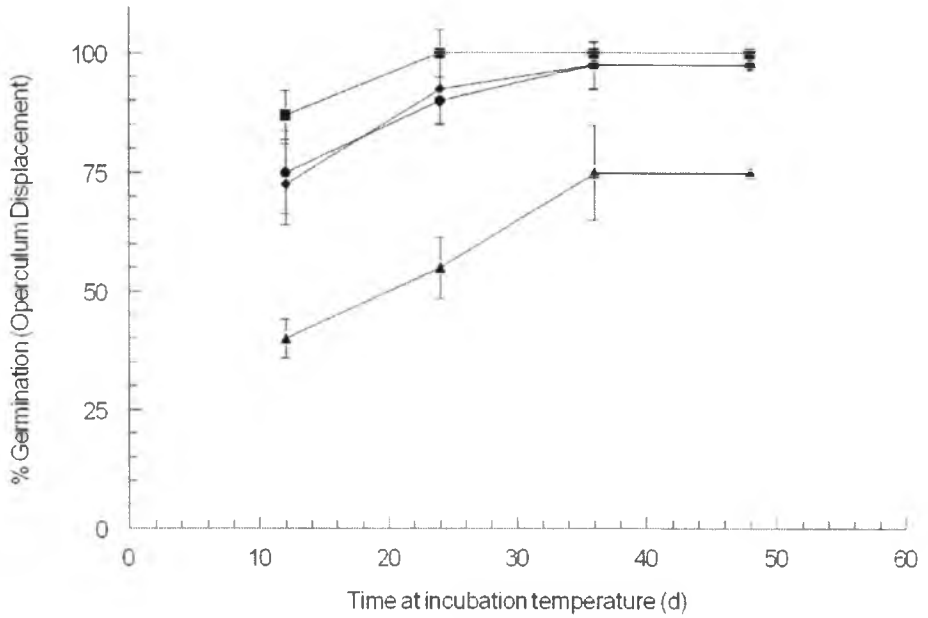

Fig. 4.9. Percentage of *Pritchardia remota* seeds with the operculum displaced after exposure to 0 (triangles), 10 (circles), 100 (diamonds), or 1000 (squares) ppm GA_3 for 24 h. Incubation was at 35°C. Error bars signify SE.

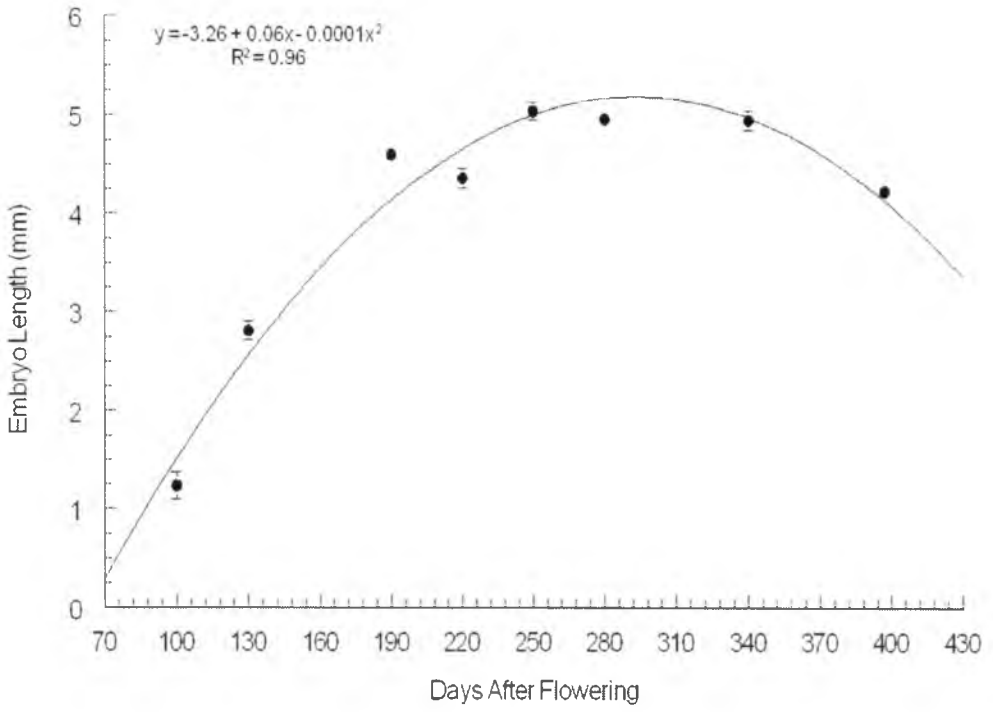

Fig. 4.10. Changes in length of developing *Pritchardia remota* embryos. Error bars are SE.

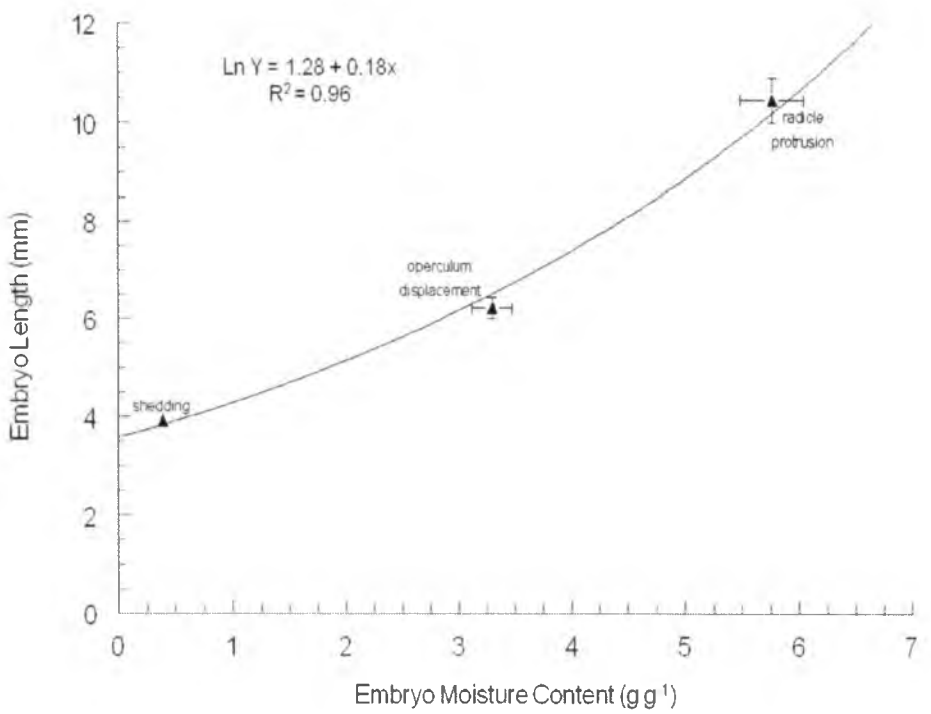

Fig. 4.11. Relationship between embryo length and moisture contents when operculum displacement and radicle protrusion in *Pritchardia remota* seeds occurred. Error bars denote SE.

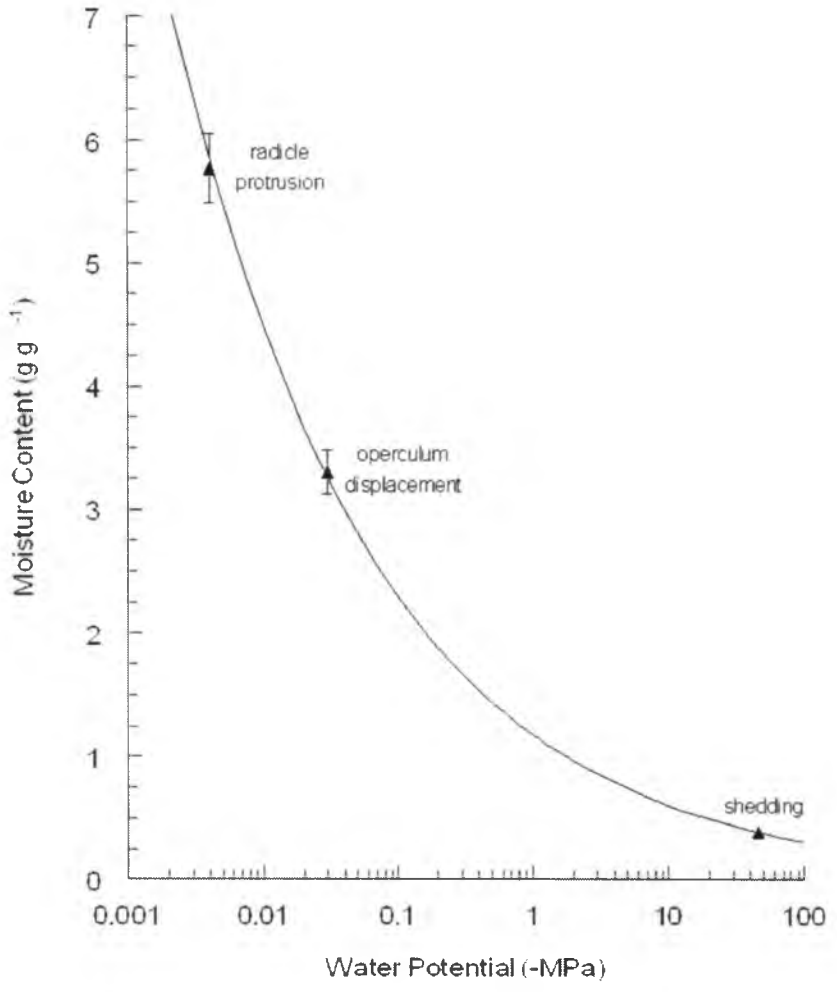

Fig. 4.12. Relationships between embryo water content and water potential during dormancy break and germination in seeds of *Pritchardia remota*. Error bars indicate SE.

CHAPTER 5

CHANGES IN OPERCULUM RESISTANCE, RESPIRATION, WATER RELATIONS AND PROTEIN PROFILES DURING DORMANCY OF *PRITCHARDIA REMOTA* (KUNTZE) BECC. EMBRYOS

Introduction

Species within Arecaceae (Palmae) are notorious for having seeds with long periods of dormancy (Uhl and Dransfield 1987; Broschat and Meerow 2000; Orozco-Segovia *et al.* 2003). Dormancy in palm seeds may be caused by impermeable coats, mechanical restriction by embryo covering structures (*i.e.* operculum and endocarp) and underdeveloped embryos (Broschat and Meerow 2000; Ehara *et al.* 2001; Orozco-Segovia *et al.* 2003). Stratification at constant warm temperatures is typically required to break dormancy and promote germination in the majority of palm species with dormant seeds (Broschat and Meerow 2000). However, there is no information on the changes in water relations, respiratory activity of embryos, or mechanical restraint of covering structures during dormancy alleviation and germination in palms. Furthermore, only limited information exists on proteins involved in palm seed germination.

Drupes of *Pritchardia remota* (Kunzte) Becc., an endangered palm endemic to Hawaii, are dormant at shedding. Dormancy mechanisms for this species include mechanical resistance of embryo growth by covering structures (*i.e.* operculum and endocarp) and underdeveloped embryos. In *P. remota*, the operculum is a lid-like portion of the seed coat that is subtended by 1- to several layers of endosperm (see Chapter 4). Presumably, pectin degrading enzymes are necessary to degrade the endosperm layer of the operculum in African oil palms (Hussey 1958).

Incubating seeds of *P. remota* at 35°C or imbibing seeds in gibberellic acid solutions alleviates dormancy and promotes germination (see Chapter 4), but physiological aspects of these treatments are not known. Therefore, the effects these treatments was invesitgated on embryo growth, respiratory activity, operculum resistance, and protein production. A Gilson Differential Respirometer was used to measure oxygen uptake. Additionally, an Instron Universal Testing Machine was used to measure the amount of force required to dislodge the operculum from seeds incubated for up to 28 d. Changes in moisture content and water potential were determined for embryos excised from seeds incubated at 15°, 25°, or 35°C for up to 28 d. Finally, we used peptide mass finger-printing to create protein profiles of treated seeds.

Materials and Methods

Plant Material

Fully mature fruits of *Pritchardia remota* from three trees growing on the grounds of the University of Hawaii at Manoa (Honolulu, Oahu) were harvested at weekly intervals by gently shaking infructescences or collecting fruits that had been shed naturally. All harvests took place from September to October 2005 and fruits from the different trees were combined. If necessary, fruits were stored in a single layer in open trays or polypropylene bags at room temperature for no more than one week.

Growth of Embryos in Water or PEG Solutions

The length of five embryos was measured with digital calipers immediately after excision in order to establish a basis for measuring growth. Embryos were then placed in 0.5 ml distilled water or PEG solutions (-0.05 to -1.45 MPa) for 48 h at 25°C. Preparation of PEG solutions was as described below. Embryos were removed from the

solutions, blotted dry, and re-measured. The percent change in length of embryos was calculated using the formula $(L_f - L_i/L_i) \times 100$; where L_f and L_i are final length and initial length, respectively.

Oxygen Consumption by Embryos at Various Temperatures

Oxygen consumption rates were used to estimate metabolic rates of mature *P. remota* embryos at the shedding stage (*c.* 397 DAF). Oxygen uptake was measured manometrically with a Gilson differential respirometer. To absorb CO_2, 1 ml of a 10% (w:v) KOH solution was injected into the central well of Warburg flasks and wicked with folded filter paper strips. Measurement of *P. remota* oxygen uptake was taken on five embryos, replicated two to three times, at 15°, 25°, and 35°C over 8 h. Oxygen uptake rates were calculated from the slopes ($r^2 \geq 0.90$) of pressure-time course data and adjusted for embryo dry weight (Walters *et al.* (2001).

Force Required to Dislodge the Operculum

Seeds were removed from mature fruits and sown as described previously (see Chapter 4). After 3, 7, 10, 14, 21, and 28 d of incubation seeds were cut into blocks approximately 6.5 x 6.5 mm. The operculum was centered on each block. Excess endosperm was removed using a cordless rotary tool (Mini-Mite 750-02, Dremel Inc., Racine, WI) set to high speed and engraving tool attachment. The blocks were about 2 mm thick after trimming, with some endosperm remaining intact. All embryo remnants were removed with a dissecting needle, leaving a cavity in the seed block.

An Instron Universal Testing Machine (Instron Engineering Corp., Canton, MA.) was used to determine the force required to dislodge the operculum immediately after sample preparation. The seed block was placed on a plastic platform with a 4-mm-

diameter counter hole drilled into it. The platform was placed on an acrylic cylinder and this apparatus was loaded onto the basal load cell. A metal rod, 1.6 mm in diameter and with a flat end, was used to approximate the portion of the embryo that comes into contact with the operculum. A drill chuck holding the metal rod was attached to the crosshead. The crosshead was lowered so that the rod entered the endosperm cavity and when activated the rod dislodged the operculum. The load cell was set to 100g (0.98 N) full scale load. Chart and crosshead speeds were 30.5 and 10.2 cm min^{-1}, respectively.

Embryo Moisture Content and Water Potential Changes During Incubation

Ninety seeds were extracted from randomly selected fruits and sown in trays as described previously (see Chapter 4 Materials and Methods). Seeds were incubated at constant 15°, 25°, or 35°C and a 12 h alternating photoperiod for 3, 7, 10, 14, 21, and 28 d. Five seeds were assigned to each treatment in a completely randomized fashion. After the appropriate incubation period, embryos were excised according to Pérez (2005) and fresh weights recorded. Each sample was dried at 95°C for 96 h, and then dry mass was determined gravimetrically. Water content was calculated from fresh and dry mass and is stated as g H_20 per g dry mass (g g^{-1}).

Water potential was calculated by constructing isotherms for embryos held at each temperature. Five replicates of one embryo each were soaked in polyethylene glycol (PEG, M_r = 8000) solutions of different concentrations ($\Psi_w \geq$ -12 MPa) for 48 h. The embryos were blotted dry after soaking, and then the fresh and dry mass of each embryo was recorded. Water content was determined as above. The water potential of different PEG solutions at different temperatures was determined according to Michel and Kaufman (1973) and verified by thermocouple psychrometry (Model SC-10,

Decagon Devices, Pullman, WA, USA). The water content and water potential of embryos from incubated seeds was related with the water sorption isotherms. Embryos displaced opercula at some incubation temperatures and times. Water content and potential were therefore calculated separately for embryo fractions that had dislodged the operculum and those that had not.

Protein Profiles

Total Protein Extraction and Gel Electrophoresis

Ten to 15 embryos were frozen in liquid nitrogen immediately after 1) excision from seeds at shedding; 2) imbibition on moist blotter for 24 h; 3) excision from seeds incubated at 15°, 25°, or 35°C for one week; and 4) excision from seeds imbibed in 1 or 10 ppm GA_3 for 24 h and stored at -80°C until processing. Total proteins were extracted from 150 mg of embryo tissue per treatment using a Plant Total Protein Extraction kit (Sigma, St. Louis, MO) according to manufacturer protocols.

One-dimensional SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). All samples (20 μg) were mixed with SDS-PAGE sample buffer and heated at 100°C for 5 min. Denatured proteins were separated on 10-20% gradient polyacrylamide SDS gels and then stained with Coomassie dye (G-250). Ten microliters of Precision Plus protein standards (Bio-Rad, Hercules, CA, USA) were applied on the gels for determination of molecular weights. Samples were dialyzed due to high lipid content. Moreover, two lanes were overloaded and subsequently these samples were diluted 10 times. Dialyzed and diluted samples were re-run as above.

Protein bands (50 kDa range) were sliced from the gel, destained with 50% (v/v) acetonitrile in 50 mM NH_4HC0_3, and completely dried in a speed-vacuum centrifuge.

Then 20 μl of sequencing-grade modified porcine trypsin (20 μg/μl in 50 mM NH_4HCO_3) was added to the dried gels slices treated with dithiothreitol and iodoacetamide prior to addition of trypsin. The unabsorbed solution was removed before 20 μl NH_4HCO_3 of was added to the rehydrated slices. These samples were incubated at 37°C overnight. Tryptic digestion was stopped by adding 5 ul of 2% trifluoroacetic acid (TFA). The digested peptides were extracted from each gel slice by sonication of 0.1% TFA and 50% acetonitrile/0.1 % TFA for 45 min. Both supernatants were combined for LC-MS/MS analysis.

Nano-electrospray LC-MS/MS Analysis

LC-MS/MS analyses were carried out with the Ultimate™ system interfaced to a quadropole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The gradient was (A = 0.1% formic acid; B = 0.1% formic acid in acetonitrile) 5% B 5 min, 60% B 88 min, 95% B 10 min, 5% B 15 min, and 5% B 20 min. Peptide spectra were recorded over a mass range of m/z 300-2500, MS/MS spectra were recorded in information dependent data acquisition over a mass range of m/z 50-1600. One peptide spectrum was recorded followed by two MS/MS spectra; the accumulation time was 1 sec for peptide spectra and 2 sec for MS/MS spectra. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Doubly or triply charged ions were selected for product ion spectra. MS/MS spectra were interpreted by Mascot (Matrix Science Ltd., London, UK) via Biotools 2.2 software (Bruker Daltronics, Billerica, MA).

Analysis of Peptide Sequences

Peptide mass fingerprint (PMF) searches based on measured peptide masses were performed using the SWISSPROT (http://ca.expasy.org/) database (Boeckmann *et al.* 2003) with the Mascot program. PMF used the assumption that peptides are monoisotopic, oxidized at the methionine residues and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. Mass tolerance of 1.0 Da was the window of error allowed for matching the peptide values. Probability based MOWSE scores were estimated by comparison of search results against estimated random match populations and were reported as: 10 x $\log_{10}(p)$, where p is the absolute probability. Scores in Mascot greater than the score at $p = 0.05$ were considered significant, meaning that for scores higher than the score at $p = 0.05$ the probability of that match being a random event is lower than 0.05. The algorithm used for determining the probability of a false-positive match with a given mass spectrum is described in Berndt (1999).

Results

Growth of Embryos in PEG Solutions

The percent increase in length of embryos held in distilled water or PEG solutions was minimal. Embryos soaked in PEG solutions greater than -0.3 MPa or distilled water increased in length by 9 ± 1.9 to 17 ± 2.5%. The increase in length of embryos placed in PEG solutions of ≤ -0.7 MPa did not exceed 5% (Fig. 5.1). In terms of dormancy relief and germination (*i.e.* radicle protrusion), the elongation of embryos observed here was not sufficient to dislodge the operculum. Germination occurs when *P. remota* embryos

increase in size by 59 and 167% for operculum displacement and radicle protrusion, respectively (see Chapter 4).

Oxygen Consumption Increases Dramatically at 35°C

Oxygen uptake rates increased dramatically when mature (397 DAF) embryos were maintained at 35°C rather than at 15° or 25°C (Fig 5.2). Metabolic rates at 35°C were 9.4- and 28.3-fold higher than at 15° and 25°C, respectively. Water content of embryos at each temperature was not significantly different ($P = 0.65$). However, metabolic rates differed significantly ($P = 0.03$) and partitioned into two homogeneous groups (Fig 5.2). Reactions occurring in *P. remota* embryos (Fig 5.3) did not follow typical Arrhenius behavior (Vertucci and Leopold 1987).

Force Required to Dislodge the Operculum

The force required to dislodge the operculum from seeds incubated at 25° and 35°C decreased rapidly as incubation time increased. Comparatively, the required force for seeds at 15°C decreased gradually (Fig 5.4). A small proportion of seeds, incubated at 35°C, were able to dislodge the operculum when its resistance was about 4.65 N, however, this amount of force was inhibitory at other incubation temperatures. Seeds incubated at 25° or 35°C had 50% or more of opercula displaced when the puncture force decreased to ≤ 3.25 N. Seeds incubated at 25°C had to be exposed one week longer than seeds at 35°C to reach this threshold puncture force (Fig. 5.4). Operculum resistance did not decrease sufficiently to allow displacement by the embryo when seeds were incubated at 15°C.

Embryo Moisture Content and Water Potential Changes During Incubation

Increases in embryo moisture content and decreases in operculum resistance were always greater for seeds incubated at 25° and 35°C than at 15°C for any given incubation time (Fig 5.5). Between 3 to 10 d of incubation embryo moisture content increased by 195, 242, and 340% at 15°, 25°, and 35°C, respectively. The increase in moisture content for embryos at 35°C was based on the proportion of embryos that had dislodged the operculum at day 10. At this time interval 40% of the embryos had not dislodged the operculum. The water content for this fraction of embryos was 2.12 ± 0.07 g g^{-1} (mean ± SE) (Table 5.1).

The decreases in operculum resistance, corresponding to the previous changes in water content, equaled 42, 72, and 71%. Although operculum resistance was similar from days 3 to 10 at 25° and 35°C, only embryos at 35°C displaced opercula during this period. Although operculum displacement occurred earlier at 35°C it was less uniform than at 25°C.

The water potential of embryos increased substantially (*i.e.* became less negative) as temperature and incubation time increased (Fig 5.6). However, these pressures could not overcome the mechanical resistance of the operculum during early stages of incubation. In fact, during incubation, embryos expand and contact the operculum, but displacement did not occur (Fig. 5.7).

The effect of temperature was most striking at 35°C, where embryo water potential was over 37-fold and 87-fold greater than at 25° and 15°C, respectively, at 3 d of incubation. Embryo water potential at 25°C was over 2-fold higher than at 15°C.

Consequently, embryos from seeds at 35°C reached higher water potentials earlier than embryos at 25 and 15°C.

Operculum displacement occurred for embryos held at 25° and 35°C when a threshold water content of 1.95 and 1.17 g g^{-1}, respectively, was surpassed. Complete operculum displacement took place when embryos had moisture contents between 3.0 and 6.0 g g^{-1} (Fig. 5.5). There was a considerable difference in the water potential threshold for the initiation and completion of operculum displacement at 35° and 25°C (Fig. 5.6). For example, operculum displacement at 35°C started after embryo water potential increased past -0.33 MPa. However, embryos at 25°C had to reach water potentials greater than -0.18 MPa before displacement occurred. Displacement for seeds at 35°C occurred after operculum resistance decreased below 5.02 N, a value that is 1.5 times greater than the threshold at 25°C (Figs. 5.5 and 5.6). Complete operculum removal occurred when the required force decreased below 2.72 and 4.14 N at 25° and 35°C, respectively.

Embryos from seeds incubated at 15°C increased in moisture content and water potential; however, no change resulted in operculum displacement. Embryos from seeds at 15°C remained at considerably lower water potentials compared to embryos from 25° and 35°C (Fig. 5.6). Water potential increased to -0.14 MPa after 28 d of incubation at 15°C. Although this water potential was adequate for operculum displacement in a fraction of seeds at higher temperatures it was inadequate at 15°C. The water potential for embryos at 28 d in the 15°C treatment corresponded to a resistance force of 3.65 N. This resistance is 10% greater than the threshold force at 25°C and sufficient to prevent displacement.

Protein Profiles

We used peptide mass fingerprinting to obtain protein profiles of *P. remota* embryos after differential treatments. As expected, proteins of various molecular weights were separated by SDS-PAGE, but only proteins in the 50 kDa range were excised due to the intense signal at this weight (Fig 5.8). The total number of proteins detected per treatment was variable. However, the greatest number of proteins was detected in embryos imbibed for 24 h or excised from seeds incubated at 25°C. The percentage of unique proteins, compared to other treatments, found in embryos imbibed for 24 h or from 25°C was 17.6 and 13.9% respectively. Embryos in the remaining treatments synthesized between 3.7 and 10.2% unique proteins. GA treated embryos had the smallest percentage of distinctive proteins. The most common proteins among treatments are displayed in Table 5.2.

Multiple isoforms of indole-3-acetic acid-amido synthetase were evident in fresh embryos and those from seeds at 25° and 15°C. These proteins were not, however, found in embryos exposed to dormancy breaking treatments. Alternatively, proteins required for DNA synthesis and transcription and cellular growth were detected in embryos imbibed for 24 h or from seeds at 35°C, 1 ppm and 10 ppm GA_3 (Appendix). One protein involved in DNA transcription was noticed in 25°C embryos. G2/mitotic-specific cyclin 2, a protein involved in regulation of the cell cycle at the G2-mitosis transition, was detected in 25°C embryos. Similarly, embryos from 15°C possessed a protein that controls the cell cycle at the G1-start transition (Appendix).

Furthermore, it is interesting to note that embryos from freshly shed seeds (*i.e.* dormant) and those exposed to dormancy-breaking treatments (35°C, 1 ppm and 10 ppm

GA_3) contained various isoforms of lipoxygenase and no antioxidant gene products from the 50 kDa range (Appendix). On the other hand, imbibed embryos contained only one lipoxygenase isoform, but also possessed L-ascorbate peroxidase 1 (cytosolic) and peroxidase 1. No lipoxygenases were detected from embryos excised from seeds incubated at cooler temperatures. However, embryos at 25° and 15°C possessed catalase isoenzyme B and glutathione reductase (cytosolic) (Appendix).

Discussion

Excised embryos of *P. remota* do not grow sufficiently to dislodge the operculum or for radicle protrusion when imbibed on distilled water or PEG solutions of low water potential. However, incubation of intact seeds at constant warm temperatures stimulates embryo growth by rapidly increasing oxygen uptake, embryo moisture content and water potential and decreasing operculum resistance. The inability of excised embryos to germinate in water or PEG solutions suggests that they are dormant (Bewley and Black 1994; Bradford 1996; Baskin and Baskin 2001; Fenner and Thompson 2006) and that dormancy is due, in part, to a physiologically inhibiting mechanism (Baskin and Baskin 2001). Then again, excised embryos had no access to storage reserves when incubated on moist blotter.

Alternatively, excised oil palm (*Elaeis guineensis* Jacq.) embryos are considered non-dormant due to "slight" elongation upon excision and imbibition on water-moistened blotter at 28°C (Hussey 1958). However, this author did not measure embryos to determine how much growth was required to dislodge the operculum. The increase in length due to re-hydration may only cause the embryo to swell to its ultimate size during development. Oil palm embryos undergo maturation drying (Aberlenc-Bertossi *et al.*

2003) that may cause shrinkage. Hussey (1958) noted that elongation of excised embryos at 25°C was very slight. However, elongation was significantly enhanced at constant temperatures between 30 to 40°C.

In the present study, we did not investigate the ability of excised embryos to grow in various osmotic solutions during and after dormancy-breaking treatments. Nonetheless, previous (see Chapter 4) and current work suggests that *P. remota* seeds exposed to 35°C had embryos that grew sufficiently to dislodge the operculum when their water potentials were above -0.77 and -0.33 MPa. This falls within the range of water potentials that allow germination in a variety of non-dormant seeds (Fyfield and Gregory 1989; Evans and Etherington 1990; Bradford 1995; Vertucci and Farrant 1995; Battaglia 1997; Bauer *et al.* 1998).

Temperature appears to have a profound effect on metabolic rates in mature, dry embryos. Increasing the temperature obviously increases the metabolic rate. However, the behavior of metabolic rates deviates from linear depicted on an Arrhenius plot, suggesting that *P. remota* embryos do not follow typical Arrhenius behavior over a wide temperature range. Additionally, at 35°C *P. remota* embryos may actually be aging rapidly. Although this treatment increased germination in dormant seeds, less vigorous seedlings were produced (see Chapter 4). If this is the case, then it may be a reason for non-linear Arrhenius behavior (Walters 1998).

This study indicated that operculum resistance decreased before embryo growth commenced. Weakening of embryo covering structures to allow germination has been shown for several species under various experimental conditions (Groot and Karssen 1987; Sánchez *et al.* 1990; Welbaum *et al.* 1995; Downie *et al.* 1997; Sánchez and de

Miguel 1997; Toorop *et al.* 1998, 2000; Amaral da Silva *et al.* 2004; Amaral da Silva *et al.* 2005). However, Liptay and Schopfer (1983) consider the force exerted by growing tomato radicles and not the resistance of endosperm caps to regulate germination. In *P. remota*, increasing incubation temperatures from 15 to 35°C and lengthening of the incubation period facilitate the decrease in operculum resistance. Similarly, the force required to penetrate *Capsicum annum* endosperms decreased rapidly as imbibition times increased and when seeds were incubated at 25°C rather than 15°C (Watkins and Cantliffe 1983). The phytochrome system is excluded as a cause for inducing weakening (Sánchez *et al.* 1986; Sánchez and de Miguel 1997) in the operculum, because *P. remota* embryos dislodge opercula equally in light or total darkness (see Chapter 4).

On the other hand, application of GA_3 enhances operculum displacement and germination in *P. remota* seeds compared to controls (see Chapter 4, Fig. 4.9). A layer of thick-walled endosperm cells subtends the operculum of *P. remota*. This layer is 1-2 cells thick at the middle of the operculum, but becomes gradually thicker towards the periphery (see Chapter 4). It is plausible that some changes must occur in these cells for a given decrease in resistance. Therefore, the regulation of operculum displacement through gibberellin-induced promotion of hydrolytic enzymes deserves further attention (Bewley 1997). Hussey (1958) surmised that, after dormancy is alleviated, embryos of oil palm secrete pectic enzymes that may be required for operculum displacement.

The endocarp and seed coat, including the operculum, of *P. remota* are heavily lignified (see Chapter 4). This may be one reason for the slow imbibition and subsequent germination of seeds. It was interesting that embryos of *P. remota* imbibed for 24 h on moist blotter synthesized Peroxidase 1 precursor (Appendix). Among many functions

this secreted protein degrades lignin (Boeckmann *et al.* 2003) and may be involved in improved imbibition or operculum weakening in *P. remota*.

Endosperm cells at the periphery of opercula in *Phoenix dactylifera* seeds are thin-walled (Gong *et al.* 2005). This finding is contrary to what is observed in *P. remota* seeds. Gong *et al.* (2005) argue that it is the anatomical structure of thin-walled endosperm cells at the periphery of the operculum and not endo-β-mannanase activity that facilitates displacement of the operculum.

Aside from causing changes in respiratory activity and operculum resistance, temperature also affects dormancy in *P. remota* seeds by promoting physiological changes in the embryo. For example, between 3 to 10 d of incubation, embryos at 15°, 25°, and 35°C are at hydration levels suitable for respiration, protein synthesis, cell division, and germination (Vertucci and Farrant 1995). Yet, over this time frame, enough growth to dislodge the operculum is manifest in a fraction of embryos only at 35°C. This may be due to the greater capacity for metabolic activity of embryos at 35°C than at 15° or 25°C (see Chapter 3).

Moreover, insufficient imbibition pressure, and possibly turgor pressure, exists early during incubation for embryos at 15° or 25°C to remove the operculum. Clearly, the rate of imbibition is increased with increasing temperatures, allowing embryos at 35°C to cross moisture content, water potential and operculum resistance thresholds earlier than seeds at lower temperatures. The dependence of increased imbibition rates on increasing temperatures has been recognized for some time (Vertucci and Leopold 1983). However, even at 35°C, not all embryos had dislodged the operculum at early sampling dates. This suggests that the remaining embryos had to cross secondary

thresholds for the completion of operculum displacement (Toorop *et al.* 1998) or that the primary threshold was reached later because of other factors delaying water uptake.

It is interesting that embryo water potential increased substantially at progressively higher temperatures and that operculum displacement occurred sooner at warmer temperatures. One explanation for the promotive effect of warm, constant temperatures is that they satisfy thermal and/or hydration time requirements sooner than at other temperatures (Bradford 1995, 2002). Moreover, the high water potentials observed may indicate that base water potentials are also being exceeded earlier; thus, dormancy alleviation (Ni and Bradford 1992; Bradford 1996).

Pritchardia remota seeds have a narrow temperature range for operculum displacement around 25°C (see Chapter 4). Exposing seeds to constant warm temperatures apparently hastens the mechanisms involved in dormancy alleviation. It is reasonable that alternating temperatures and soil moisture, as found in the natural habitat, slow the accumulation of required thermal or hydration time by dormant embryos (Finch-Savage *et al.* 1999). Eventually, however, these requirements are met for some seeds in the population and germination can proceed in certain seed fractions over time.

By only investigating protein profiles from bands with the most intense signals the possibility of detecting the multitude of gene products involved in dormancy-break and germination was excluded (Gallardo *et al.* 2001); including lower molecular weight cell-wall degrading enzymes (Groot and Karssen 1987; Bewley *et al.* 1997; Downie *et al.* 1997). Nevertheless, the protein profiles discussed here reflect dormancy patterns in *P. remota* embryos. For example, multiple isoforms of indole-3-acetic acid-amido

synthetase were detected only in fresh embryos or embryos excised from seeds incubated at 25° or 15°C.

These proteins are reported to catalyze the synthesis of IAA-amino acid conjugates as a mechanism to cope with excess auxin (Boeckmann *et al.* 2003), implying that auxin concentration may be high in dormant embryos. Auxin is synthesized in stem and root apices. This phytohormone is known to affect several aspects of growth and development including cell elongation, cellular differentiation and root initiation (Hopkins 1995; Zubay 1998). A high auxin concentration can inhibit root growth (Hopkins 1995), and this may be one reason why embryos are physiologically dormant at shedding. In *P. remota* embryos, the IAA-synthetases may function to ensure a proper balance of auxin and cytokinins that will favor root over shoot development once embryos overcome multiple dormancy mechanisms.

Alternatively, gene products involved in DNA synthesis and transcription are found mostly in embryos exposed to dormancy-breaking treatments. This finding suggests that cells receiving this type of treatment are proceeding rapidly through the cell cycle and embryos may begin growing by cell division. The sizes that embryos must attain in order to dislodge the operculum, break through the endocarp, and eventually germinate (*i.e.* radicle protrusion) (see Chapter 4), lends weight to the hypothesis that embryo growth must be a combination of cell enlargement and cell division. Applying external stimuli to alleviate dormancy, therefore, hastens DNA synthesis and transcription, hence embryo elongation.

Protein products involved in DNA synthesis, replication, and transcription were not found in fresh embryos, but were observed in embryos imbibed for 24 h. This

suggests that upon adequate re-hydration cellular events leading to DNA synthesis and repair may occur. However, since embryos are metabolically quiescent at shedding the DNA specific proteins observed in imbibed embryos are most likely involved in transitioning embryos from a G_0 to the early G_1 phase. During this phase extensive DNA repair must take place before resumption of the cell cycle (Kozeko and Troyan 2000; Baskin and Baskin 2001; Osborne *et al.* 2002; Vázquez-Ramos and de la Paz Sánchez 2003). This phase may be drawn-out in drupes of *P. remota* as imbibition was shown to be slow (see Chapter 4).

In embryos exposed to dormancy-breaking treatments no cell-cycle-regulating proteins (*e.g.* cyclins) were detected; implying that these embryos are physiologically competent to overcome dormancy and continue with germination. Alternatively, proteins known to control the cell cycle (Gallardo *et al.* 2001; Osborne *et al.* 2002; Vázquez-Ramos and de la Paz Sánchez 2003) were found in imbibed embryos held at 15° and 25°C (Appendix). Apparently, embryos from 25°C have cells further along in the cell cycle than those at 15°C. For instance, cell cycle control is exerted at the G2/M phase in 25°C embryos by a B-like cyclin. At 15°C cell cycle regulation seems to occur at an earlier stage due to a G1/S-specific cyclin. Therefore, at 25° and 15°C *P. remota* embryos can not break dormancy due to regulation of the cell cycle by specific proteins. In embryos held at 35°C cyclins may have been destroyed (Vázquez-Ramos and de la Paz Sánchez 2003) thus allowing for rapid initiation of cell division.

Embryos must sense the appropriate conditions for alleviation of dormancy and germination, leading to advancement of the cell cycle (Finch-Savage *et al.* 1998; de Castro *et al.* 2001). An incubation temperature of 15°C is not appropriate for dormancy

relief in *P. remota* and seems to maintain the cell cycle at an early stage. This may also be a result of embryonic hydration levels (Osborne *et al.* 2002). Embryos incubated at 15°C for 7 d had a water potential of -3.65 MPa. Nucleic acid repair and protein synthesis are not predicted to occur at this hydration level (Vertucci and Farrant 1995). At the same time, embryos incubated at 25° and 35°C are at hydration levels high enough to allow these processes to occur (see Fig. 3). Although, the water potential of embryos at 25°C may be adequately low to limit cell cycle activities during the first days of incubation.

Lipoxygenases (LOX) have received much attention in the seed biology literature. However, the roles of LOX remain controversial (Loiseau *et al.* 2001; Bailly *et al.* 2002). On the one hand, LOX are known to catalyze the hydroperoxidation of fatty acids. This reaction produces reactive oxygen species that may further degrade membranes and other macromolecules, thereby facilitating ageing (St. Angelo *et al.* 1979). However, the presence of LOX, or its isoforms, does not necessarily indicate that deterioration occurs because of these enzymes (Sung and Jeng 1994; Bailly *et al.* 2002). LOX may be involved in development, mobilization of storage lipids, protection from pathogens, cell elongation, and improved hydration (Loiseau *et al.* 2001; Bailly *et al.* 2002).

The role of LOX in *P. remota* embryos in not known, but synthesis patterns among treatments suggest that these enzymes may be involved in dormancy alleviation. For example, isoforms of LOX were found predominately in embryos given dormancy breaking treatments or fresh embryos, but not in other treated embryos (Appendix). What's more no gene products, from the 50 kDA range, involved in free-radical or peroxide scavenging were evident in embryos under dormancy-breaking conditions. The

lack of protective enzymes in fresh embryos can be explained by their metabolic quiescence; although mature *P. remota* embryos retain antioxidant capacity at shedding (see Chapter 3).

However, if LOX are detrimental, why are there no protective mechanisms present in embryos exposed to dormancy-breaking treatments? Embryos imbibed for 24 h had one type of LOX, but two types of antioxidant proteins. No LOX were detected in embryos from 25° and 15°C, but embryos from each temperature displayed the presence of protective enzymes. Moreover, seeds with high unsaturated fatty acid content, particularly linoleic and linolenic acid, are susceptible to oxidative degradation by LOX (St. Angelo *et al.* 1979; Priestley 1986; Bailly *et al.* 2002). *P. remota* embryos are lipid rich (Pérez and Walters, unpublished data). For example, the total lipid content of embryos is 58% unsaturated fatty acids, of these 33% are linoleic acid. This suggests that embryos would be open to degradation by LOX. Yet, drupes remain viable, albeit dormant, for some time (see Chapter 4). Tests with drupes of other *Pritchardia* species indicate that they can remain viable in the soil for up to a year. Therefore it does not appear that LOX are detrimental to the viability of *P. remota* embryos.

The evidence presented here indicates that *P. remota* embryos are dormant at shedding. In order for dormancy to be alleviated, embryos must first generate enough pressure to dislodge the operculum, and then enough to rupture the endocarp (see Chapter 4). This implies that the cell cycle must be active for necessary growth. Embryos that can attain high water potentials sooner will alleviate dormancy and germinate faster. Constant temperatures greater than or equal to 25°C can facilitate these processes by increasing the rate of metabolism. At lower temperatures, thresholds in required force,

water potential, and cell cycle activities are breached later and thus embryos are incompetent to alleviate their morpho-physiological dormancy at an earlier time.

Tables

Table 5.1. Percentage of incubated seeds with opercula intact and the corresponding embryo water content and water potential.

Treatment	Operculum Intact %	Moisture Content g g^{-1} Average ± SE	Water Potential (MPa) Average ± SE
25°C			
14d	20	2.07 ± 0	-0.14 ± 0
35°C			
7d	60	1.18 ± 0.13	-0.04 ± 0.02
14d	40	2.12 ± 0.07	-0.02 ± 0.01

Table 5.2. Common proteins, identified by peptide mass fingerprinting, in *P. remota* embryos after excision from freshly shed fruits (Fresh); imbibition for 24 h; excised from seeds incubated for at 15°, 25°, or 35°C for one week; and excised from seeds imbibed in solutions of 1 or 10 ppm GA_3 for 24 h. + and – denote presence or absence respectively.

	Treatment						
Protein Name	Fresh	Water 24 h	35°C 1 week	25°C 1 week	15°C 1 week	ppm GA_3 1	10
26S protease regulatory subunit 7	-	-	-	+	-	+	-
DNA directed RNA polymerase beta" chain	-	+	+	+	-	-	-
DNA polymerase alpha catalytic subunit	-	+	-	-	-	+	-
DNA polymerase delta catalytic subunit	-	+	+	-	-	+	+
Lipoxygenase 2	-	-	-	-	-	+	+
Lysyl-tRNA synthase	-	-	-	-	-	+	+
Nitrate reductase (NADH) 1	-	+	-	-	-	+	-
Probable indole-3-acetic acid-amido synthetase GH3.5 & GH3.8	-	+	-	+	+	-	-
Putative lipoxygenase 3	-	+	+	-	-	+	-
Serine/theroine-protein kinase SAPK4	+	-	-	-	-	+	-
Transcription factor RF2b	-	+	+	-	-	-	-

Figures

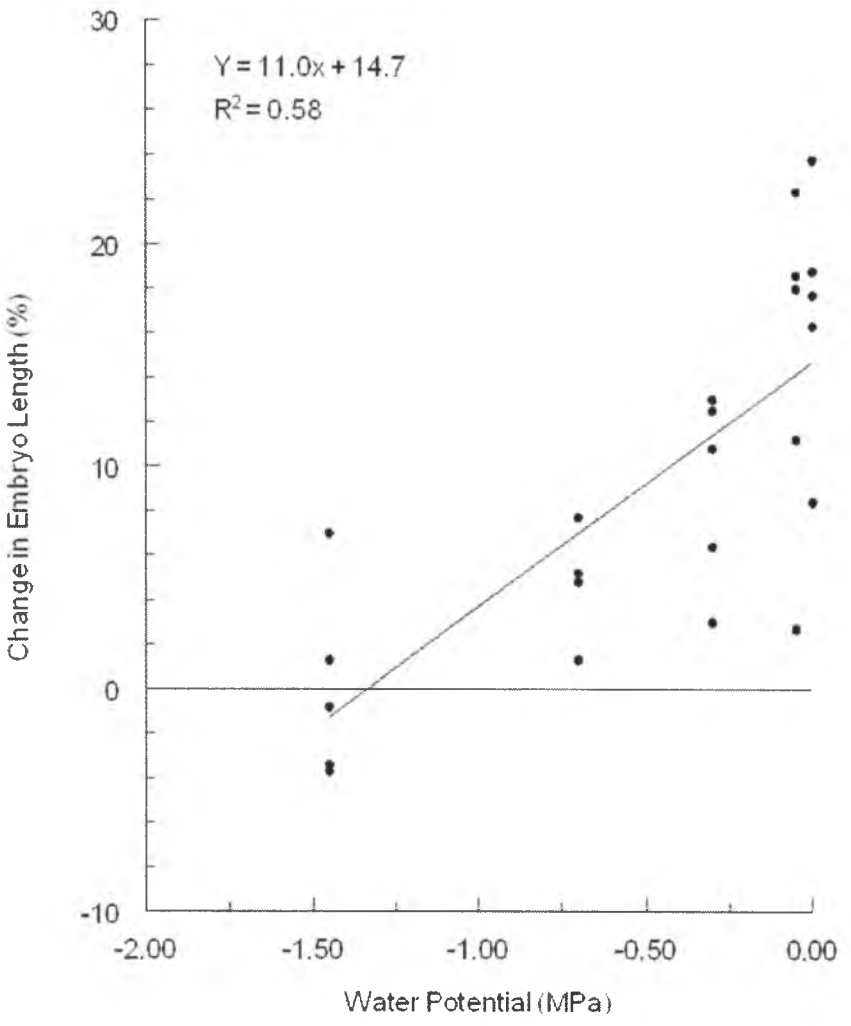

Fig. 5.1. Relationship of percentage change in length of *Pritchardia remota* embryos after imbibition for 48 h in PEG solutions of various water potentials.

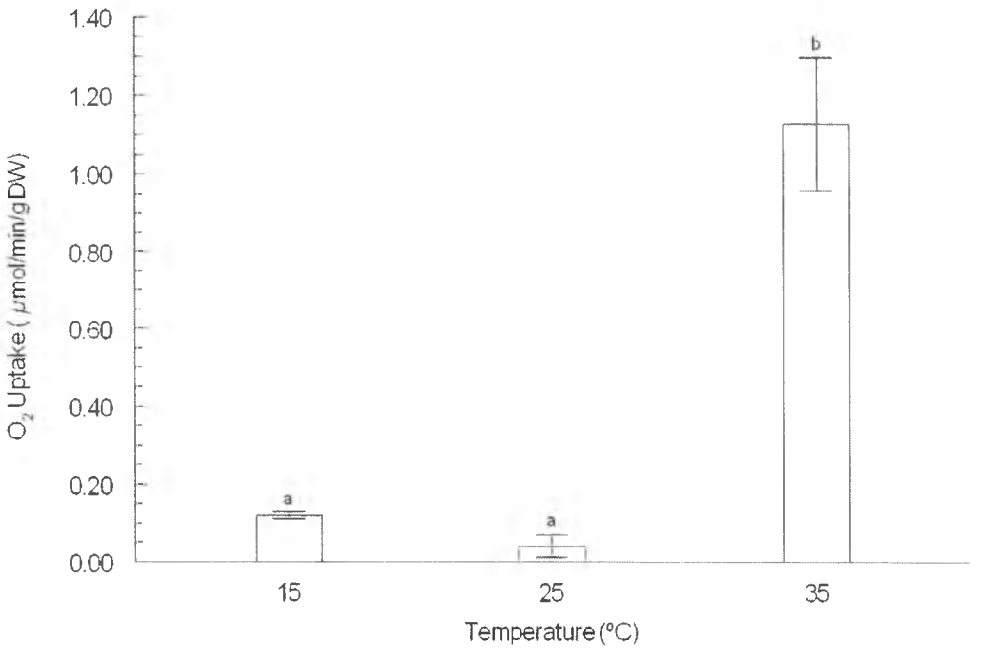

Fig. 5.2. Oxygen uptake rates of mature (397 DAF) embryos exposed to constant 15°, 25°, or 35°C. Bars with the same letters were not significantly different at $\alpha = 0.05$ level. Mean separation was by Duncan's Multiple Range test. Embryo water content among treatments was not significantly different ($P = 0.65$). Error bars denote SE.

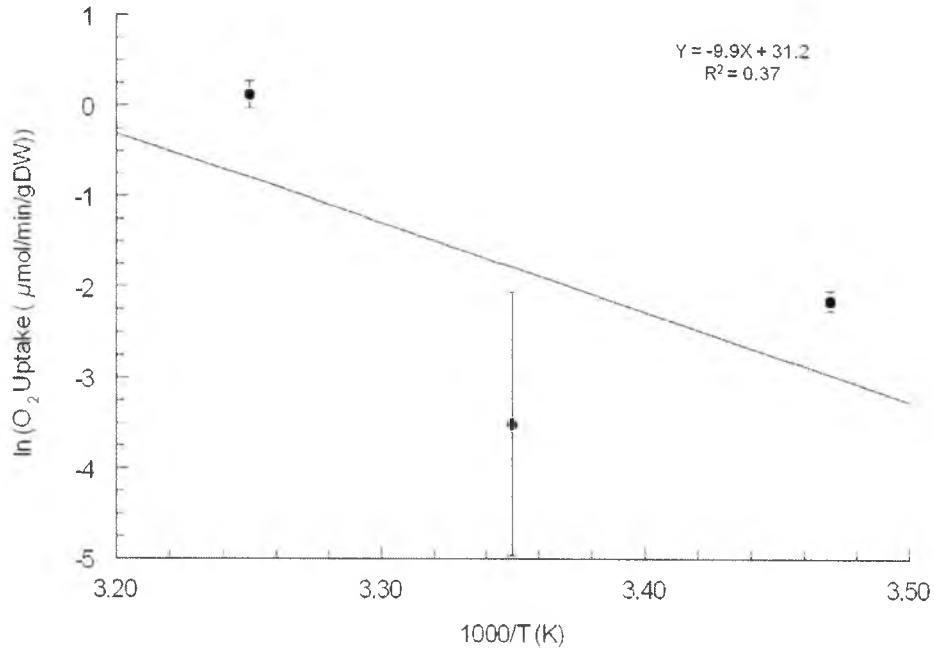

Fig. 5.3. Arrhenius plot for mature (397 DAF) *Pritchardia remota* embryos exposed to 15°, 25°, or 35°C. Error bars denote SE.

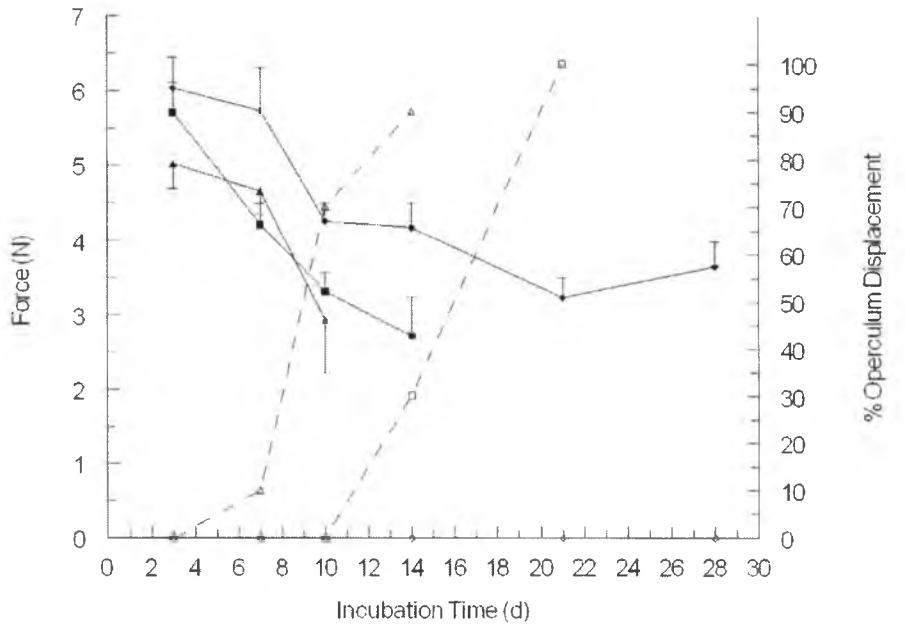

Fig. 5.4. Force in Newtons required to dislodge the operculum from seeds that had been incubated at 35°C (triangles), 25°C (squares), or 15°C (diamonds). Open symbols represent the fractions of seeds that had displaced operculum at each sampling date. Force measurements were taken on seeds with intact opercula for each sampling date. Error bars denote SE.

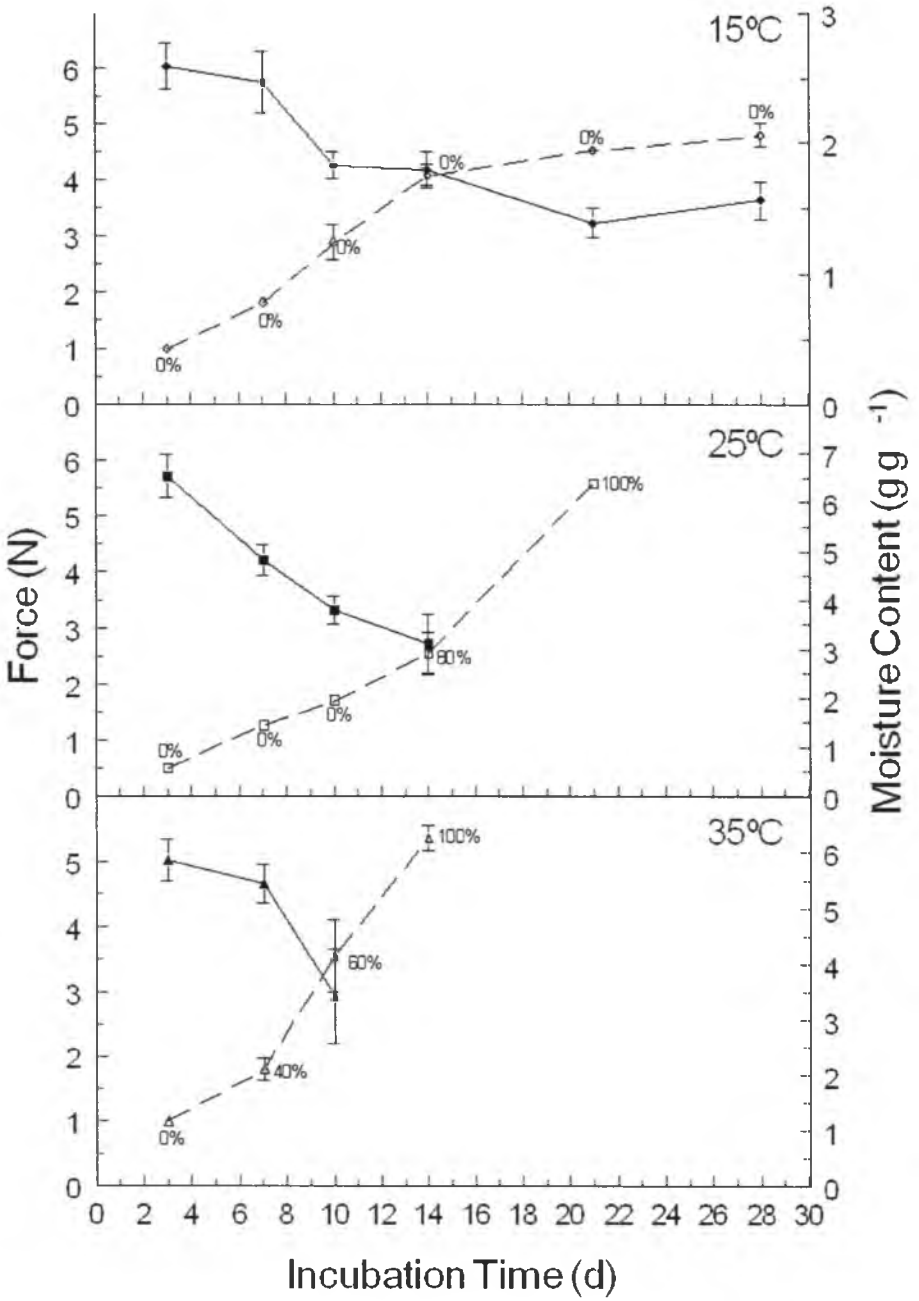

Fig. 5.5. Changes in required puncture force (closed symbols) and embryo moisture content (open symbols) during incubation at 15°, 25°, or 35°C. Percentages refer to percent operculum displacement during incubation. Error bars signify SE.

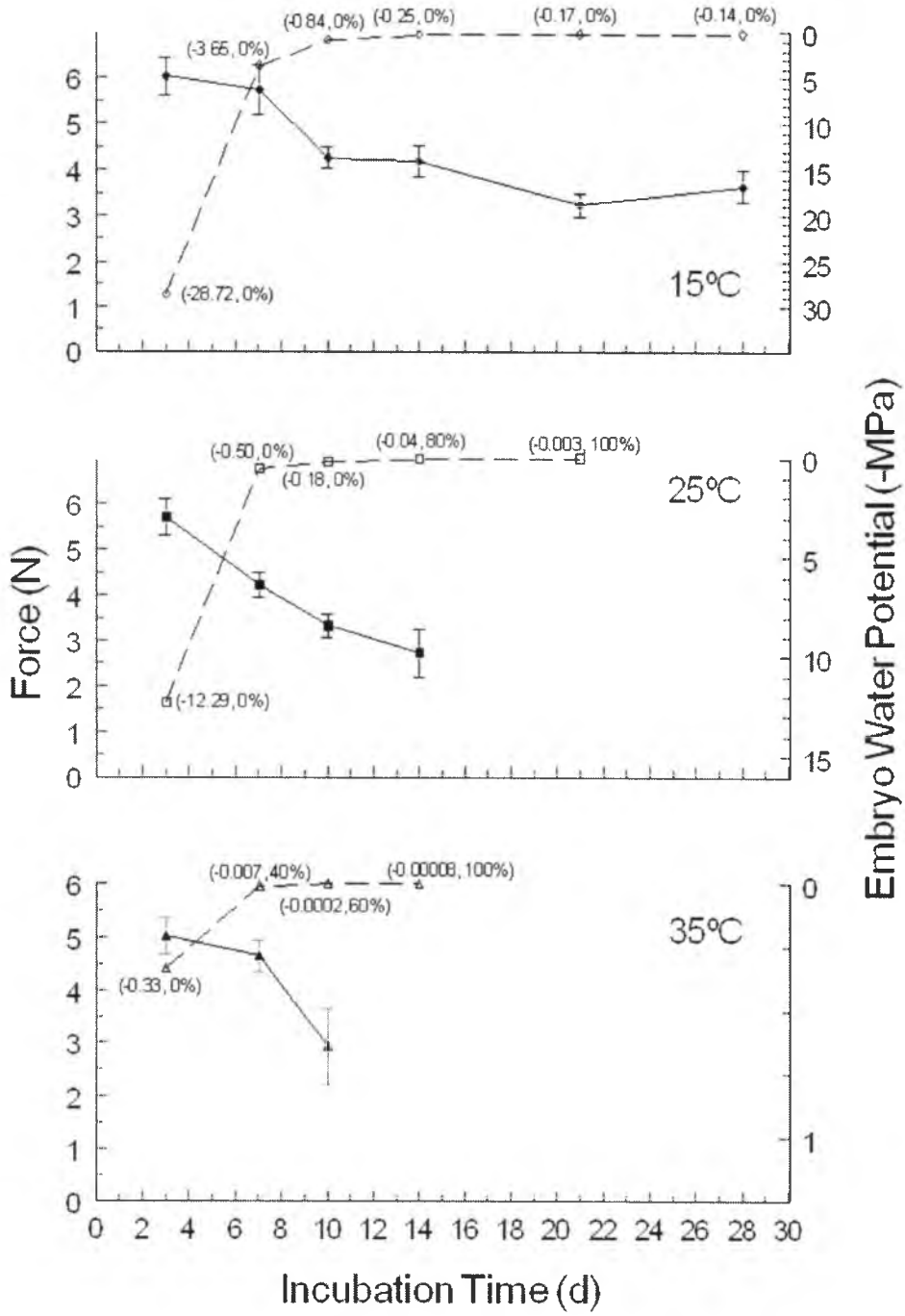

Fig. 5.6. Changes in required puncture force (closed symbols) and embryo water potential (open symbols) during incubation at 15°, 25°, or 35°C. Values in parentheses denote water potential and percent operculum displacement during incubation. Error bars signify SE.

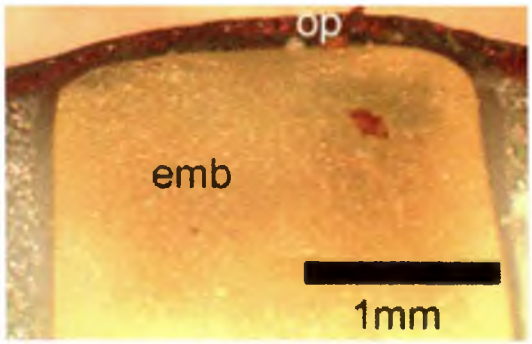

Fig. 5.7. A *Pritchardia remota* embryo from seeds held at 25°C for 10 d. Note that the embryo has expanded and is contacting the operculum but has not dislodged it. OP = operculum, emb = embryo, 2.5x.

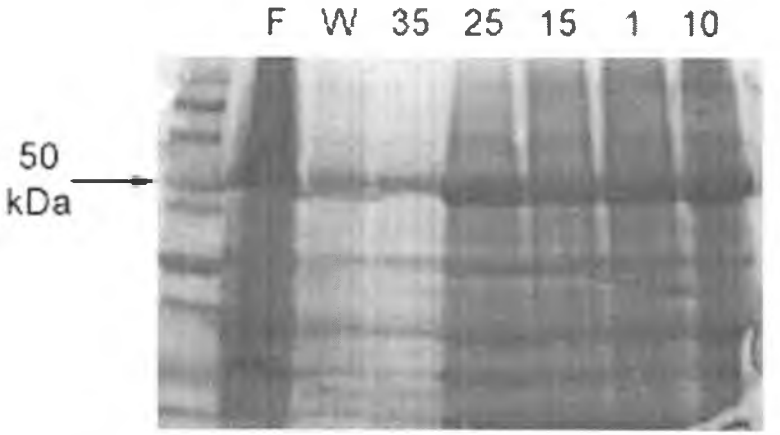

Fig. 5.8. Separation of total *Pritchardia remota* embryonic proteins by SDS-PAGE gel electrophoresis. Note intensity of band in the 50 kDa range. F = fresh embryos, W = excised embryos imbibed on moist blotter for 24 h., 35, 25, and 15 represent embryos excised from seeds incubated at 35°, 25°, and 15°C for one week, 1 and 10 denote embryos excised from seeds imbibed in 1 or 10 ppm GA_3 for 24 h.

CHAPTER 6

DAMAGED SEEDS OF TWO RARE HAWAIIAN PALMS (*PRITCHARDIA*, ARECACEAE) RETAIN THE ABILITY TO GERMINATE: IMPLICATIONS FOR SEED PREDATION

Introduction

Seed predators are known to limit recruitment of various species. Recruitment depression of *Picea mariana* (Côte *et al.* 2003), *Quercus liaotungensis* (Li and Zhang 2003) and many species indigenous to New Zealand, including one palm (Campbell and Atkinson 2002), has been attributed to seed predation by rodents. Rats (*Rattus* spp.) are predators of seeds of various plant species throughout the South Pacific (Allen *et al.* 1994; McConkey *et al.* 2003). In Hawaii, limited recruitment of endemic *Pritchardia* (Arecaceae) species is anecdotally linked to seed predation by introduced rats.

The black rat (*Rattus rattus*) is implicated as the primary consumer of dispersed fruits and seeds of *Pritchardia kaalae* Rock (Arcand *et al.* 2003), a federally listed endangered species (U.S.F.W.S. 2003). Middens of partially eaten fruits and seeds have been found within the *P. kaalae* population (Pérez, pers. obs.). However, it is not known if partially eaten seeds of *Pritchardia* retain the ability to germinate and become established.

Different amounts of seed damage can limit germination and cause recruitment limitation of some plant species. For example, increasing endosperm damage, as a result of feeding by curculionid beetle larvae, significantly decreased percent germination in acorns of *Quercus alba* and *Q. rubra* (Weckerly, *et al.* 1989). Similarly, final germination percent decreased in seeds of *Lupinus perennis* when 30 and 60% of

endosperm tissue was surgically removed (Zhang and Maun 1991). The number of germinating *Vicia sativa* seeds decreased significantly as damage by tortricid moth larvae increased from 0-75% by volume (Koptur 1998). Moreover, delayed germination was reported in seeds of *Agropyron psammophilum* and *Elymus canadensis* after surgically removing 30 and 60% of the endosperm (Zhang and Maun 1991).

Conversely, germination rate and percentage may be enhanced as a result of partial endosperm removal. Significantly faster germination (*i.e.* decrease in mean time to complete germination) was found in *V. sativa* when seed damage was either less than 10% or 10-25% by volume; compared to control, 25-50% and 50-75% damage (Koptur 1998). Germination of *Calamovilfa longifolia* and *Leymus arenarius* was accelerated, relative to control seeds, when 30% and 60% of seed reserves were surgically removed (Zhang and Maun 1991).

In this study, we sought to determine whether germination of *P. kaalae* (endangered) or *Pritchardia hillebrandii* (Kuntze) Becc. (species of conservation concern) is affected by partial removal of endosperm. *Pritchardia* fruits are single seeded drupes (Fig. 6.1). We tested the hypotheses that 1) final germination percentage (FGP) decreases and 2) mean time to complete germination (MTG) increases with increasing levels of endosperm removal. To test these hypotheses, varying amounts of endosperm were removed with horticultural pruners from seeds of both species.

Materials and Methods

Fruit collection occurred between July and September, 2002. Ripe fruits of *P. hillebrandii* were harvested randomly from nine cultivated trees growing in Kalaheo, Kauai (21°54'N, 159° 35'W). Fruits of *P. kaalae* were collected from 57 accessible trees

of the wild population growing within steeply graded, lowland mesic forest on the Ohikilolo Ridge, Oahu (21°30'N, 158°11'W, elev. 933m). Fruits were stored in open trays on a laboratory bench-top (Honolulu, HI) at about 25°C. Experimentation commenced approximately 3 weeks after collection. Seeds were extracted from the pericarp then soaked in a 70% (v:v) ethanol solution for 5 min followed by a 20 min soak in 0.5% (v:v) sodium hypochlorite.

Four levels of endosperm removal (0.0, 12.9, 42.5 and 75.2% by volume) were applied randomly to seeds of each species. Seeds are typically spherical at maturity. *Pritchardia kaalae* seeds are larger (7.0 g/seed, dry mass basis; 10.1 cm^3/seed) than those of *P. hillebrandii* (1.1 g/seed, dry mass basis; 0.8 cm^3/seed). Over 99.0% of the seed volume is comprised of endosperm; the embryo occupies about 0.3% of seed volume (Fig. 6) (Pérez, unpublished data).

To keep the embryo intact, endosperm removal began on the hemisphere opposite the operculum. Seed with no visible operculum were excluded, as they do not possess an embryo (Pérez, pers. obs.). For greater accuracy of final measurements, a cordless Dremel Rotary Tool (Model 750, Dremel, Racine, WI) with a small engraving cutter, set at low speed, was used to remove finer amounts of endosperm.

A combination of morphological (underdeveloped embryo) and physiological (physiological inhibiting mechanism) dormancy (Baskin and Baskin 2001) is found in seeds of *Pritchardia* (see Chapters 4 and 5). To overcome these dormancy mechanisms, all seeds were incubated at constant 30°C with a 12-h photoperiod for 35 to 49 days in clear, plastic trays (15 x 12.5 x 4.5 cm) filled with moistened sphagnum moss that had been previously sterilized in an autoclave. For each species four experimental units of 15

seeds were blocked on possible light and temperature gradients within the germination chamber.

After the third day of incubation, fungal contamination was observed in the sphagnum moss of some experimental units. As a result, all seeds were sprayed with a 1% (w:v) solution of N-(trichloromethyl) thiol)–4–cyclohexane-1, 2-dicarboximide (captan). Seeds were sprayed again after day 24 of incubation. Germination counts occurred every week and stopped 1 week after the last observed germination. We considered germination to have taken place when the embryo dislodged the operculum (Fig. 6.1).

Mean time to germination was calculated using the following formula: MTG = $\Sigma (f \cdot x)/ \Sigma f$; where f is the number of seeds germinated on day x and x is the number of days after sowing (Furutani *et al.* 1985). Percent germination data were arcsin transformed. Conclusions did not change as a result of transformation; therefore, analyses of untransformed data are presented. We performed analysis of variance on FGP and MTG using the GLM procedure (SAS 2001). Mean separation was by Duncan's Multiple Range test at $\alpha = 0.05$ level.

Results

The average final germination percentage (FGP) for *P. hillebrandii* and *P. kaalae* remained above 75% or any volume of endosperm removal between 0 and 42.5%. However, germination dropped below 50% when about ¾ of the endosperm volume was removed (Fig. 6.2A, C). FGP for *P. kaalae* at the 75% level of endosperm removal equaled 20 ± 7.2% (mean ± SE). Although FGP decreased for *P. hillebrandii* with increasing levels of endosperm removal, ANOVA detected no differences in the FGP for

this species (Table 6.1), most likely due to the high amount of variation in germination (CV = 29.5). Alternatively, FGP in seeds of *P. kaalae* was significantly different (Table 6.1) at the highest level of endosperm removal compared to low or moderate levels of removal (Fig. 6.2C).

Mean time to germination, for both species, was greatest when endosperm remained intact (Fig. 6.2B, D). Treated seeds of either species germinated approximately two times faster, on average, than control seeds. Moreover, germination at any level of endosperm removal was always more rapid for the smaller seeds of *P. hillebrandii* than for the larger seeds of *P. kaalae* (compare Fig. 6.2B and D). For both species, means separated into two homogenous groups according to Duncan's Multiple Range test (Fig. 6.2B, D).

Discussion

The ability of *P. hillebrandii* and *P. kaalae* seeds to germinate to relatively high proportions is retained, in a species-specific manner, as endosperm is removed. Moreover, seed damage hastens germination compared to non-damaged seeds. No significant reduction in percent germination occurred when approximately one-eighth (12.9%) to almost one-half (42.5%) of endosperm volume was removed. Likewise, Steele *et al.* (1993) found that half-eaten *Quercus phellos* acorns retained their ability to germinate. *Prioria copaifera*, a large-seeded tropical species, did not suffer reduced probability of germination compared to untreated seed when 60.0% of its cotyledonary mass was removed (Dalling *et al.* 1997). Another large-seeded tropical species, *Gustavia superba*, was able to germinate and establish with 50% of cotyledonary reserves removed (Dalling and Harms 1999).

The difference in FGP observed between the species tested suggests that *P. kaalae* is more tolerant to loss of nearly half its endosperm volume than *P. hillebrandii*. On the other hand, seeds of *P. hillebrandii* may be more tolerant of higher levels of endosperm damage than those of *P. kaalae*. The hypothesis that FGP decreases as endosperm removal increases could not be supported for *P. hillebrandii* most likely due to a high degree of variation in germination. However, the hypothesis that germination decreases for endosperm removal levels greater than 42.5% for is supported for *P. kaalae*.

A similar decrease in MTG occurred for *P. hillebrandii* and *P. kaalae* after 0, 12.9, 42.5, and 75.2% endosperm removal. Suggesting that mechanisms involved in controlling germination rate may be the same for both species. However, more rapid germination in *P. hillebrandii* may be due to its smaller seed mass. Water may penetrate a seed with less endosperm mass more rapidly, especially when it is damaged, than a seed with larger endosperm mass, resulting in decreased MTG. The evidence for neither species supports the hypothesis that MTG increases with increasing levels of endosperm removal. In fact the most rapid germination occurred in damaged seeds.

Clearly, germination in *P. hillebrandii* and *P. kaalae* was not limited by low to moderate levels of endosperm removal. These results imply that, in addition to morpho-physiological dormancy, the seed coat may hinder germination by exerting mechanical resistance. Additionally, the endosperm and seed coat have been shown to impede imbibition. Mechanical scarification did not improve the rate of imbibition in *P. remota* seeds or endocarps (see Chapter 4). Therefore, water penetrates *Pritchardia* seeds

slowly, even when the endocarp or seed coat has been breached. Massive damage to the seed is necessary to allow rapid penetration of water, hence decreased MTG.

Slowing the rate of imbibition until abundant soil moisture is present could be adaptive. Fruits of *Pritchardia* are shed during the dry season (May-September) in Hawaii and although sporadic rains do occur during this time, soil moisture may be limiting for seed germination and seedling development. Thus, if *Pritchardia* seeds in the wild survive low to moderate levels of damage and produce roots, conditions in the rhizosphere, especially moisture, may not be favorable for continued growth. The early seedling stage of *Pritchardia* palms is very susceptible to being killed by low soil moisture (Pérez, pers. obs.).

It is plausible then that limited damage, although not directly responsible for mortality, may still lead to recruitment failure by breaking dormancy in seed of *P. hillebrandii* and *P. kaalae* during a season when soil moisture is not adequate for seedling establishment. Seedlings of *P. hillebrandii* and *P. kaalae* from damaged seeds had less developed roots systems and smaller eophylls than those from undamaged seeds (Pérez, pers. obs.). Alternatively, fungal contamination could attack a damaged seed more easily than an intact one. Intact seeds have been shown to maintain the ability to germinate even after heavy fungal contamination to the fruit coat (see Chapter 4). However, predators may irreparably damage embryos during feeding.

Rapid and complete germination following damage could instead be an adaptive response. Damage could somehow signal germination to take place, as moisture can better penetrate the seed. Thus, seedlings could rapidly establish and, assuming non-

limiting soil moisture and adequate food reserves for root and leaf development, avoid seed death by germinating before fungal damage to injured tissues becomes extensive.

Tables

Table 6.1. ANOVA tables for the final percent germination and mean time to complete germination in days of *Pritchardia hillebrandii* and *Pritchardia kaalae* seeds after removal of 0, 12.9, 42.5, or 75.2% of endosperm volume. Seeds were incubated at constant 30°C for 35 to 49 d.

Final Germination Percent					
Species	Source	df	MS	*F*	*P*
P. hillebrandii	Trt	3	0.20	3.54	0.06
	Blk	3	0.09	1.52	0.28
	Err	9	0.06		
P. kaalae	Trt	3	0.52	54.90	<0.0001
	Blk	3	0.02	2.14	0.17
	Err	9	0.08		
Mean Time to Complete Germination (d)					
P. hillebrandii	Trt	3	40.09	9.84	0.003
	Blk	3	2.35	0.58	0.65
	Err	9	4.08		
P. kaalae	Trt	3	210.27	25.26	0.0001
	Blk	3	4.83	0.58	0.64
	Err	9	8.32		

Figures

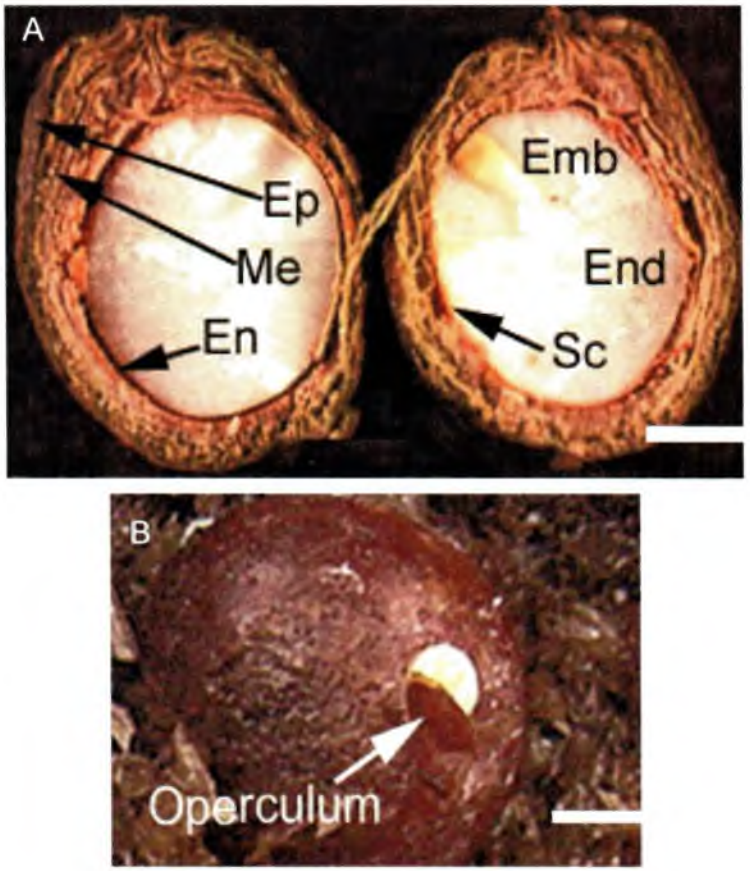

Fig. 6.1. (A) Cross section of mature *P. kaalae* fruit showing fruit coat, seed and embryo. The fruit of *Pritchardia* is a single seeded drupe. The fruit coat (pericarp) consists of the outer epicarp (Ep), middle fibrous layer, or mesocarp (Me), and inner, woody layer, or endocarp (En). The seed consists of the hard seed coat (Sc), the white, hard endosperm (End) and the underdeveloped embryo (Emb). Scale bar 5mm. (B) Germinating embryo of *P. kaalae* forcing operculum aside. Scale bar = 3mm.

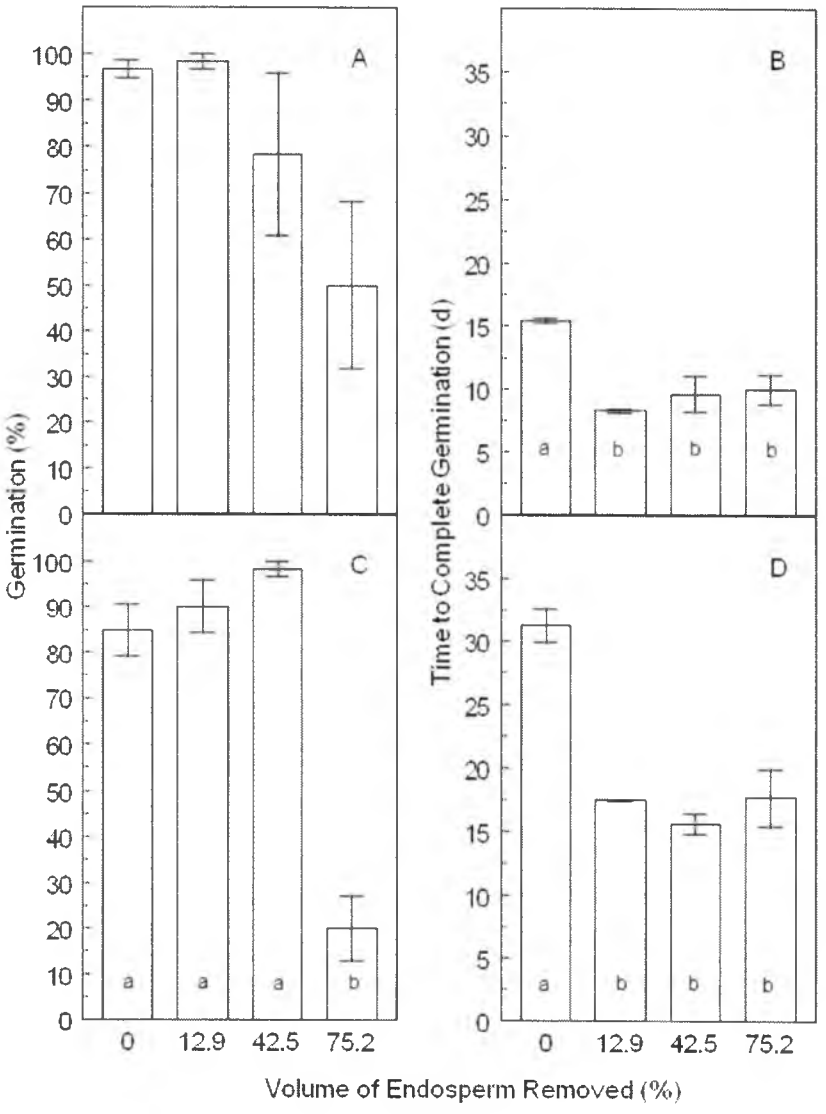

Fig. 6.2. Final percent germination (A, C) and mean time to complete germination in days (B,D) of *Pritchardia hillebrandii* (A, B) and *Pritchardia kaalae* (C, D) seeds after removal of 0, 12.9, 42.5, or 75.2% of endosperm volume. Error bars denote SE. Columns with the same letters are not significantly different at α = 0.05 according to Duncan's Multiple Range test.

CHAPTER 7

CONCLUSIONS

In conclusion, developmental patterns in *P. remota* are intermediate to those observed for species that can and can not tolerate storage under genebank conditions, implying that embryos of this species may be stored *ex situ*. However, the viability of intermediate tissues is known to be of limited duration under standard genebank conditions. Additionally, although embryos do tolerate relatively high levels of drying and remain viable, they are not able to withstand the extreme drying necessary to maintain viability in genebanks. Moreover, further post-harvest drying may force embryos deeper into a dormant state. This may confound germination tests of tissues that have been stored. Therefore, it may be necessary to employ other techniques, such as cryo-preservation, to extend the longevity of this species in storage.

Dormancy in *P. remota* is caused by a combination of factors. First embryo growth is restricted by the operculum and endocarp. Second, embryos are underdeveloped and must reach a critical length, moisture content, and water potential for operculum displacement and subsequent radicle protrusion. *Pritchardia remota* drupes and seeds are capable of imbibition and are therefore not physically dormant. Germination is hastened at warm constant temperatures (*i.e.*, $\geq 25°C$) and if seeds are removed from the fruit coats. The warm temperatures may stimulate imbibition and metabolic activities that lead to rapid cell division and embryo growth.

Although not tested on *P. remota*, moderate levels of seed damage may not be limiting in terms of germination for *Pritchardia* species. On the contrary, damage to the endosperm caused seeds to germinate more rapidly compared to non-damaged seeds.

Nevertheless, damaged seeds may succumb to fungal attack. Or, if prompted to germinate early, seedlings may die due to lack of sufficient soil moisture. It is critical therefore to prevent predators from preying upon *Pritchardia* seeds.

Appendix

Proteins, with significant Mascot scores, identified by peptide mass fingerprinting searches on the SWISSPROT or MSDB databases for *P. remota* embryos upon: immediate excision from seeds (fresh); after imbibition on moist blotter for 24 h; excision from seeds after incubation at 15, 25 or 35°C for 1 week; and excision from seeds imbibed in 1 or 10 ppm GA_3 for 24 h. Peptides were separated and analyzed by LC-MS/MS.

Treatment	Protein Name	Synonym	Accession Code	Mascot Score	Probable Function
Fresh					
	Asparagine synthetase	Glutamine-dependent asparagine synthetase	Q43011	20(18)	
	Indole-3-acetic acid-amido synthetase	Auxin-responsive GH3-like protein 1	Q8LQM5	19(18)	Catalyzes synthesis of IAA-amino acid conjugates, mechanism to cope with surplus auxin
	Probable Lipoxygenase 4	Carotene oxidase Lipoperoxidase Lipoxidase	Q53RB0	33(18)	Involved in growth and development, pest resistance, and senescence or responses to wounding. Catalyzes hydro-peroxidation of lipids
	Putative Lipoxygenase 5	Carotene oxidase Lipoperoxidase Lipoxidase	Q7XV13	18(18)	Same as above
	Methionyl-tRNA synthetase	Methionine-tRNA ligase	Q9ZTS1	27(18)	
	Nicotianamine synthase 2	S-adenosyl-methionine 3-amino-3-carboxypropyltransferase 2	Q9FEG8	25(18)	Synthesis of nicotianamine, iron sensing

	Potassium transporter 3	OsHAK3	Q5ZC87	20(18)	High-affinity potassium transporter
	Serine/theroine -protein kinase SAPK4	Osmotic stress/ABA-activated protein kinase 4	Q5N942	28(18)	Signal transduction in osmotic stress
	Sucrose synthase 3	Sucrose-UDP glucosyltransfe rase 3	Q43009	23(18)	Provides UDP-GLU and FRU through SUC cleaving
Embryo Imbibition 24 h					
	26S protease regulatory subunit 4 homolog	TAT-binding protein homolog 2	P46466	32(18)	ATP-dependent degradation of ubiquitinated proteins
	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	ACCase beta chain	P12218	20(18)	Fatty acid biosynthesis
	Cation transporter, HKT4	OsHKT4	Q7XPF8	20(18)	Regulation of potassium and sodium homeostasis
	DNA directed RNA polymerase beta' chain	PEP, Plastid-encoded RNA polymerase beta' subunit RNA polymerase beta' subunit	P12092	21(18)	Catalysis of DNA transcription into RNA using the four NTP's as substrates
	DNA directed RNA polymerase beta" chain	PEP, Plastid-encoded RNA polymerase beta" subunit RNA polymerase beta" subunit	P12093	31(18)	Catalysis of DNA transcription into RNA using the four NTP's as substrates

DNA polymerase alpha catalytic subunit	DNA nucleo-tidyltransferase (DNA directed) DNA-dependent DNA polymerase	O48653	38(18)	DNA replication
DNA polymerase delta catalytic subunit	DNA nucleo-tidyltransferase (DNA directed) DNA-dependent DNA polymerase	Q9LRE6	49(18)	DNA synthesis and degradation of single stranded DNA
Elongation factor G, mitochondrial precursor	mEF-G	Q9FE64	19(18)	GTP-dependent translocation of proteins on ribosome
Ferrochelatase II, chloroplast precursor	Protoheme ferro-lyase Heme synthetase	O22101	19(18)	Catalysis of ferrous insertion into protoporphyrin
L-ascorbate peroxidase 1, cytosolic	APXa	P93404	21(18)	Removal of hydrogen peroxide from chloroplasts and cytosol
Malate dehydrogenase, glyoxysomal precursor	Malic dehydrogenase	Q42972	20(18)	
NAC domain-containing protein 68	ONAC068	Q52QH4	22(18)	
Nitrate reductase, [NADH] 1	NR1	P16081	19(18)	Nitrate assimilation

Non-symbiotic hemoglobin 4	rHb4, ORYsa GLB1d	Q94FT7	19(18)	Oxygen sensing and electron transfer
Peroxidase 1 precursor	Lacto-peroxidase Myelo-peroxidase	P37834	27(18)	Removal of hydrogen peroxide, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin oxidative stress. A secreted protein
Phytochrome B		P25764	24(18)	Induction of various morphogenic responses in active form (viz. Pfr)
Phytoene dehydrogenase, chloroplast precursor	Phytoene desaturase	Q9ZTN9	24(18)	Conversion of phytoene to zeta-carotene
Probable auxin efflux carrier component 1c	OsPIN1c	Q67UL3	26(18)	Component of auxin outflow carrier
Probable indole-3-acetic acid-amido synthetase GH3.8	Auxin-responsive GH3-like protein 8, OsGH3-8	Q7XIN9	24(18)	Coping mechanism, through catalysis of IAA synthesis, for presence of excess auxin

Probable indole-3-acetic acid-amido synthetase GH3.12	Auxin-responsive GH3-like protein 12, OsGH3-12	Q53P49	20(18)	Coping mechanism, through catalysis of IAA synthesis, for presence of excess auxin
Probable potassium Transporter 16	OsHAK16	Q84MS3	19(18)	High-affinity potassium transporter
Putative GDP-L-fucose synthase 2	GDP-4-keto-6-deoxy-D-mannose-3, 5-epimerase-4-reductase 2	Q67WR5	27(18)	Conversion of GDP-4-dehydro-6-deoxy-D-mannose to GDP-fucose
Putative lipoxygenase 3	Carotene oxidase, Lipoperoxidase Lipoxidase	Q7G794	21(18)	Involved in growth and development, pest resistance, and senescence or responses to wounding. Catalyzes hydro-peroxidation of lipids
SCAR-like protein 2		Q5QNA6	21(18)	Regulation of actin and microtubule organization
Sucrose-phosphate synthase	UDP-glucose-fructose-phosphate, glucosyltransfe rase	Q43802	21(18)	Regulation of carbon partitioning, regulation of sucrose synthesis

	Soluble starch synthase 2-1, chloroplast precursor	Soluble starch synthase II-1	Q7XE48	46(18)	Starch synthesis in endosperm amyloplasts, deposition of starch in chloroplasts
	Transcription factor RF2b		Q6S4P4	18(18)	Vascular development, shoot tissue organization
35°C 1 week					
	60S ribosomal protein L10-3	QM/R22	Q40649	24(18)	
	60S ribosomal protein L10-1	Putative tumor suppressor SC34	P45635	22(18)	
	CIPK-like protein 1	OsCK1	Q6X4A2	19(18)	Interaction with CBL proteins
	DNA-directed RNA polymerase alpha chain	PEP, Plastid-encoded RNA polymerase alpha subunit RNA polymerase alpha subunit	P12090	20(18)	Catalysis of DNA transcription into RNA using the four NTP's as substrates
	DNA-directed RNA polymerase beta' ' chain	PEP, Plastid-encoded RNA polymerase beta" subunit RNA polymerase beta" subunit	P12093	22(18)	Catalysis of DNA transcription into RNA using the four NTP's as substrates
	DNA polymerase delta catalytic subunit	DNA nucleo-tidyltransferase (DNA directed) DNA-dependent DNA polymerase	Q9LRE6	26(18)	DNA synthesis and degradation of single stranded DNA

Inositol-3-phosphate synthase	Myo-inositol-1-phosphate synthase, MI-1-P synthase IPS	O64437	19(18)	
Lipoxygenase I	9-lipoxygenase r9-LOX1	Q76I22	30(18)	Introduction of molecular oxygen exclusively into the C-9 position of linoleic and linolenic acid
Nucleoside diphosphate kinase 1	Nucleoside diphosphate kinase I, NDK I NDP kinase I, NDPK I	Q07661	21(18)	Major role in synthesis of NTP's other than ATP, associated with microtubules
Phospholipase D alpha 2	PLD alpha 2 Choline phosphatase 2 Phosphatidylcholine-hydrolyzing phospholipase D 2	P93844	22(18)	Hydrolyzes glycerol-phospholipids at the terminal phosphor-diesteric bond. Plays an important role in various processes.
Putative lipoxygenase 3	Carotene oxidase Lipoperoxidase Lipoxidase	Q7G794	20(18)	Involved in growth and development, pest resistance, and senescence or responses to wounding. Catalyzes hydro-peroxidation of lipids

	Putative UDP-arabinose 4-epimerase	UDP-D-xylose 4-epimerase 2, UDP-galactose 4-epimerase-like protein 2 OsUEL-2	Q8H0B6	18(18)	
	Splice isoform 1 calreticulin precursor		Q9SLY8	26(18)	Molecular calcium binding chaperone promoting folding, oligomeric assembly
	Splice isoform 1 GIGANTEA protein		Q9AWL7	26(18)	Regulation of circadian rhythm, activates Hd1-CONSTANS gene.
	Telomerase reverse transcriptase	OsTERT	Q8LKW0	35(18)	Replication of chromosome termini, elongation of telomeres
	Transcription factor RF2b		Q6S4P4	20(18)	Vascular development, shoot tissue organization
25°C 1 week					
	26S protease regulatory subunit 7	26S proteasome subunit 7 26S proteasome AAA-ATPase subunit RPT1 Regulatory particle triple-A ATPase subunit 1	Q9FXT9	36(19)	ATP-dependent degradation of ubiquitinated proteins

Catalase isoenzyme B	CAT-B	P55309	21(19)	Protect cells from the toxic effects of hydrogen peroxide
Chitin inducible gibberellin-responsive protein 2		Q8GVE1	20(19)	Regulatory role in the early step of oligosaccharide elicitor response
Chloroplast 50S ribosomal protein L2		P17351	21(19)	
DNA-directed RNA polymerase beta " chain	PEP, Plastid-encoded RNA polymerase beta" subunit RNA polymerase beta" subunit	P12093	26(19)	Catalysis of DNA transcription into RNA using the four NTP's as substrates
G2/mitotic-specific cyclin-2	B-like cyclin CycOs2	Q40671	21(19)	Control of the cell cycle at the G2/M (mitosis) transition
Major pollen allergen Ory s 1	Ory s I	Q40638	26(19)	
NADP-dependent malic enzyme	NADP-ME	P43279	19(19)	
Nicotianamine synthase 1	S-adenosyl-L-methionine, S-adenosyl-methionine 3-amino-3-carboxy-propyl-transferase 1 OsNAS1	Q9SXQ7	19(19)	Synthesis of nicotianamine, iron sensing

Nonspecific lipid-transfer protein	LTP 5	O65091	22(19)	Transfers phospholipids as well as galactolipids across membranes. May play a role in wax or cutin deposition in the cell walls of expanding epidermal cells and certain secretory tissues.
Phytochrome A		P10931	25(19)	Induction of various morphogenic responses in active form (viz. Pfr)
Probable indole-3-acetic acid-amido synthase GH3.5	Auxin-responsive GH3-like protein 5, OsGH3-5	Q6I581	23(19)	May catalyze the synthesis of indole-3-acetic acid (IAA)-amino acid conjugates, providing a mechanism for the plant to cope with the presence of excess auxin

Proteasome subunit alpha type 3	20S proteasome alpha subunit G, 20S proteasome subunit alpha-7	Q9LSU0	21(19)	Cleaves peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH
Protein kinase G11A		P47997	23(19)	Regulation of metabolism and signal transduction processes
Splice isoform 1 Putative ion channel DMI1, chloroplast precursor		Q5N941	29(19)	
Splice isoform 1 Zinc finger CCCH type domain containing protein ZFN-like 2		Q5JLB5	29(19)	
Sucorse synthase 3	Sucrose-UDP glucosyltransfe rase 3	Q43009	20(19)	Sucrose-cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways

15°C 1 week					
	Calcium-dependent protein kinase, isoform 1	CDPK 1	P53682	28(19)	May play a role in signal transduction pathways that involve calcium as a second messenger.
	Fructose-bisphosphate aldose, chloroplast precursor	ALDP	Q40677	27(19)	Carbohydrate degradation, glycolysis
	G1/S-specific cyclin C-type		P93411	25(19)	Control of the cell cycle at the G1/S (start) transition.
	Glutamine synthetase shoot isozyme, chloroplast precursor	Glutamate--ammonia ligase Clone lambda-GS31	P14655	22(19)	Light-modulated chloroplast enzyme, responsible for reassimilation of ammonia generated by photo-respiration.
	Glutathione reductase cystolic	GR, GRase	P48642	19(19)	Maintains high levels of reduced glutathione in the cytosol.
	NAC domain-containing protein 71	ONAC071	Q53NF7	27(19)	
	NAD(P) H-quinone oxireductase chain K, chloroplast	NAD(P)H dehydrogenase, chain K, NADH-plastoquinone oxidoreductase subunit K	P12159	23(19)	

Oleosin 18 kDa	OSE721	Q40646	22(19)	Structural role to stabilize lipid bodies during desiccation of the seed by preventing coalescence of the oil. May also provide recognition signals for specific lipase anchorage in lipolysis during seedling growth.
Probable cation transporter HKT6	OsHKT6	Q6H501	21(19)	Probable cation transporter. May be involved in regulation of K(+)/Na(+)
Probable indole-3-acetic acid-amido synthetase GH3.8	Auxin-responsive GH3-like protein 8, OsGH3-8	Q7XIN9	20(19)	May catalyze the synthesis of indole-3-acetic acid (IAA)-amino acid conjugates, providing a mechanism for the plant to cope with the presence of excess auxin
Probable indole-3-acetic acid-amido synthetase GH3.9	Auxin-responsive GH3-like protein 9, OsGH3-9	Q6ZLA3	32(19)	Same as above
Putative indole-3-acetic acid-amido synthetase GH3.10	Auxin-responsive GH3-like protein 10, OsGH3-10	Q6ZLA7	19(19)	Same as above

	Splice isoform two-component response regulator-like PRR73	Pseudo-response regulator 73, OsPRR73	Q689G4	22(19)	Controls photoperiodic flowering response. Seems to be one of the components of the circadian clock.
	Telomerase reverse transcriptase	OsTERT	Q8LKW0	22(19)	Replication of chromosome termini, elongation of telomeres
GA_3 1ppm 24 h					
	26S protease regulatory subunit 7	26S proteasome subunit 7 26S proteasome AAA-ATPase subunit RPT1 Regulatory particle triple-A ATPase subunit 1	Q9FXT9	39(19)	ATP-dependent degradation of ubiquitinated proteins
	ASC1-like protein 1	*Alternaria* stem canker resistance-like protein 1	Q6EUN0	22(19)	Mediates resistance to sphinganine-analog mycotoxins (SAMs) by restoring sphingolipid biosynthesis.
	DNA polymerase alpha catalytic subunit	DNA nucleotidyltransferase (DNA directed) DNA-dependent DNA polymerase	O48653	23(19)	DNA replication

DNA polymerase delta catalytic subunit	DNA nucleo-tidyltransferase (DNA directed) DNA-dependent DNA polymerase	Q9LRE6	21(19)	DNA synthesis and degradation of single stranded DNA
Lipoxygenase 2	Lipoxygenase L-2	P29250	41(19)	Involved in growth and development, pest resistance, and senescence or responses to wounding. Catalyzes hydroperoxidation of lipids
Lysyl-tRNA synthase	Lysine-tRNA ligase, LysRS	Q6F2U9	22(19)	
NADH-ubiquinone oxidoreductase 27 kDa subunit	Complex I-27KD Cl-27KD, NADH dehydrogenase subunit 9	Q35322	20(19)	Transfer of electrons from NADH to the respiratory chain
Nitrate reductase, [NADH] 1	NR1	P16081	22(19)	Nitrate assimilation
Putative lipoxygenase 3	Carotene oxidase Lipoperoxidase Lipoxidase	Q7G794	21(19)	Involved in growth and development, pest resistance, and senescence or responses to wounding. Catalyzes hydroperoxidation of lipids
Serine-threonine-protein kinase SAPK4	Osmotic stress/abscisic acid-activated protein kinase 4	Q5N942	36(19)	Signal transduction in osmotic stress

	Telomerase reverse transcriptase	OsTERT	Q8LKW0	31(19)	Replication of chromosome termini, elongation of telomeres
	Tubulin gamma-2 chain	Gamma-2 tubulin	O49068	21(19)	Minus-end nucleation of microtubule assembly
	Two-component response regulator-like PRR95	Pseudo-response regulator 95, OsPRR95	Q689G6	23(19)	Controls photoperiodic flowering response. Seems to be one of the components of the circadian clock.
GA_3 10ppm 24 h					
	Delta 1-pyrroline-5-carboxylate synthetase	P5CS	O04226	32(19)	Plays a key role in proline biosynthesis, leading to osmo-regulation in plants.
	DNA polymerase delta catalytic subunit	DNA nucleo-tidyltransferase (DNA directed) DNA-dependent DNA polymerase	Q9LRE6	32(19)	DNA synthesis and degradation of single stranded DNA
	Hypothetical protein P0001A07.13		Q9FRA7	21(19)	

Lipoxygenase 2	Lipoxygenase L-2	P29250	29(19)	Involved in growth and development, pest resistance, and senescence or responses to wounding. Catalyzes hydro-peroxidation of lipids
Lysyl-tRNA synthase	Lysine-tRNA ligase, LysRS	Q6F2U9	20(19)	
Nonspecific lipid-transfer protein 2	nsLTP2, 7 kDa lipid transfer protein	P83210	20(19)	Transfer lipids across membranes. May play a role in plant defense or in the biosynthesis of cuticle layers.
Probable lipoxygenase 8	Carotene oxidase Lipoperoxidase Lipoxidase	Q84YK8	20(19)	Involved in growth and development, pest resistance, and senescence or responses to wounding. Catalyzes hydroperoxidation of lipids
Protein PAIR1	Homologous pairing aberration in rice meiosis 1 protein	Q75RY2	21(19)	Establishment of homologous chromosome pairing in early meiosis.

Putative 30S ribosomal protein S31	Putative 30S ribosomal protein S31, chloroplast [Precursor] [Fragment]	P47909	20(19)	Controls photoperiodic flowering response. Seems to be one of the components of the circadian clock.
Splice isoform Two-component response regulator-like PRR73	Pseudo-response regulator 73, OsPRR73	Q689G4	30(19)	
Sucrose-phosphate synthase	UDP-glucose-fructose-phosphate, glucosyl-transferase	Q43802	34(19)	Regulation of carbon partitioning, regulation of sucrose synthesis
Telomerase reverse transcriptase	OsTERT	Q8LKW0	20(19)	Replication of chromosome termini, elongation of telomeres

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